IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of	:	
MORISHITA ET AL.	:	GROUP ART UNIT: 1636
Serial No. 09/029,497	:	
Filed: June 9, 1998	:	EXAMINER: R.Schwartzman
For: MEDICAMENT CONTAINING HGF GENE	:	

D E C L A R A T I O N

Honorable Commissioner of
Patents & Trademarks
Washington, D. C. 20231

Sir:

I, Ryuichi MORISHITA, a Japanese citizen, c/o 11-22-502, Miyahara 2-chome, Yodogawa-ku, Osaka-shi, Osaka 532 Japan, declare and state as follows:

1. I am the first inventor of the above-identified application.

2. An up to date copy of my curriculum vitae is attached hereto. As can be seen from the curriculum vitae, I currently hold the positions of Chief of the Section of Gene Therapy, Department of Geriatric

Medicine, Osaka University Medical School, and Chief of the Section of Cardiovascular Medicine, Division of Gene Therapy Science, Osaka University Medical School. I obtained the qualification of MD from Osaka University Medical School in Japan in 1987, and the qualification of PhD from Osaka University Medical School in Japan in 1991. I am an author of 145 scientific publications. My research interests include a gene therapy using a HGF gene.

3. I am familiar with the prosecution history of the above-identified application. I have read the Final Official Action dated August 11, 1999 on the application, and the prior art references cited therein.

4. I have noted the Examiner's position in the Final Office Action that the claims are broadly drawn to any type of expression vectors comprising a hepatocyte growth factor (HGF) gene and any route of administration of the expression vector to treat a patient. Also, I have noted the Examiner's position that the claimed liposome comprising an expression vector containing the HGF gene and fused to Sendai virus and the treatment method using the liposome would be obvious over Thierry et al. or Isner each in view of Morishita et al.

5. However, as discussed in detail below, I believe that the specification provides sufficient enablement disclosure for direct administration of any type of expression vectors containing the HGF gene to a target site or injured tissue, and for intramuscular administration of any type of the expression vectors. Further, I believe that the direct administration or intramuscular administration of the expression vector for treating a subject in need of HGF is unobvious over any of the prior art references.

The Present Invention

6. I have been involved with research on the feasibility of a gene therapy using a HGF gene for many years, and have found that such a gene therapy can be applied to the treatment of various diseases. Recently, the gene therapy using the HGF gene has reached the stage of its application to clinical trial. Thus, according to the present invention, I have gained the assurance that the HGF gene would enable the gene therapy, and such my assurance is now going to be confirmed in the clinical trial stage. In Japan, many major news papers have interested in and recently

announced our clinical trial plan for the gene therapy using the HGF gene. The copies of the Japanese News Papers are attached hereto together with the English translations
thereof.

7. The present invention has, for the first time, ascertained the feasibility of the gene therapy using the HGF gene. In other words, it is not too much to say that my basic concept for the potential gene therapy using the HGF gene has been realized and completed according to the present invention.

8. At the time when the Japanese applications, based on which the present application claims the priority under the Paris Convention, were filed in Japan, it had already been confirmed and well acknowledged in the art that the HGF protein is effective in the treatment of various diseases. However, nothing whatsoever had been made clear for the feasibility of the gene therapy using the HGF gene. Indeed, it had been entirely unclear whether the HGF gene after transfected would be expressed to produce the HGF protein, and whether the HGF gene would be effective as a gene therapy. The HGF protein is composed of 728 amino acids

and thus has a high molecular weight of about 100 kDa, and has a complicated structure consisting of α-subunit and β-subunit. Further, the HGF protein can exhibits its activity only after the single chain HGF protein expressed has been cleaved to the double chain protein. From these unique characteristics of the HGF protein, it has been entirely unpredicted whether the HGF gene after transfected would be correctly expressed to produce the single chain protein followed by the processing to the active double chain protein to effectively exhibit its pharmacological activity.

9. I and my co-researchers have first investigated whether the HGF gene could function similarly to the HGF protein after transfected to vascular endotherial cells and vascular smooth muscle cells. In fact, as demonstrated by the <u>in vitro</u> tests at Test Examples 2 - 7 of the present specification, we have made clear that the HGF gene was correctly expressed after transfected to the cells and could function similarly to the HGF protein to stimulate the growth of vascular endotherial cells without replication of vascular smooth muscle cells. Furthermore, we have revealed that the HGF gene could more significantly stimulate the growth of vascular endotherial cells than

the HGF protein, as demonstrated at Test Example 2 of the specification.

10. Then, we have conducted in vivo tests to investigate the effect of the HGF gene. More specifically, as demonstrated at Test Example 8 of the specification, we have made clear that, when the HGF gene was administered to rat at the heart muscle, the gene could expressed to produce the HGF protein effectively exhibiting the angiogenesis activity, well reflecting the results from the in vitro tests at Test Examples 2 - 7 of the specification. As stated in the Amendment filed on June 30, 1999 in response to the Office Action dated December 30, 1998 on the above-identified application, the cells and animal models used in Test Examples 2 - 8 are well recognized in the art as correlating with a human diseases. Accordingly, the results from Test Examples 2 - 8 have revealed that the HGF gene is applicable to the treatment of arterial disease.

11. In the meantime, it has been already made clear and confirmed that the HGF protein is effective in the treatment of various diseases, as described in detail in the specification at pages 1 to 2. It is well

known in the art that the functions of the HGF protein is mediated by a HGF receptor, c-Met. That is, the HGF protein binds to the c-Met receptor to exhibit its biological activities such as cell growth stimulation on vasculars and tissues in various organs including liver, kidney, epithelium, brain nerve, lung and cartilage. Thus, the biological various activities of the HGF protein on various vasculars and tissues are based on the common mechanism via the c-Met receptor.

12. As stated hereinabove, the results from Test Examples 2 - 8 of the specification have, for the first time, revealed that the HGF gene is applicable to the treatment of arterial disease. Thus, from the finding that the HGF gene is applicable to the treatment of at least one disease such as arterial disease, we could have then hightly expected that the HGF gene might be similarly effective in the treatment of other diseases, namely, the HGF gene might effectively function against other diseases based on the common mechanism via the c-Met receptor and therefore might be effective in the treatment of the other diseases for which the HGF protein is effective.

13. Under this expectation, as shown in Test Example 9 of the specification, we have investigated the biological action of the HGF gene on cartilage cells, which is considered to be definitely different from the angiogenesis activity of the HGF gene. In fact, we have elucidated the cartilage repair effect of the HGF gene when administered to a rat model with injured joint cartilage, and confirmed that the administration of the HGF gene has expectedly resulted in the significant repaire of the injured joint cartilage.

14. From the results of Test Examples 2 - 9 of the specification, I have gained the assurance that the HGF gene would be effectively applicable to the treatment of almost of the diseases for which the HGF protein is effective. In this regard, the specification states at page 9, lines 8 - 10 as follows:

-- The "pharmaceutical composition" used in the present invention indicates a medicament for the treatment or prevention of human diseases, which is attributed to the pharmacological activities of HGF. For example, exemplified are medicaments for the treatment or prevention of the diseases given hereinabove. According to the present

invention, the HGF gene is introduced into cells wherein HGF is expressed in those cells to exhibit the pharmacological actions. Thus, the medicament of the present invention is effectively applicable to the diseases for which HGF itself is effective. --

15. After the present application was effectively filed in the U.S.A., some articles reporting experimental data confirming the effects of the gene therapy using the HGF gene according to the present invention have been published, as listed below:

① Ueda et al., Supplement to Circulation, Vol.96, No.8, October 21, 1997, Abstract from the 70th Scientific Session, I - 619.

This article reports that the gene therapy using the HGF gene was effective for ischemia-reperfusion injury in the heart.

② Ueki et al., Nature Medicine, Vol.5, No.2, February 1999, 226 - 230.

This article reports that the gene therapy using the HGF gene was effective for liver cirrhosis.

③ Yaegashi et al., The Welfare Ministry Specific Diseases, Respiratory Diseases Research Group, Pervaded Lung Disease Sub-group, 1997 Research Report, 51 - 53.

This article reports that the gene therapy using the HGF gene was effective for injured lung.

④ Ueda et al., Ann. Thoracic Surgeons, 1999; 67: 1726 - 1731.

This article reports that the gene therapy using the HGF gene was effective for ischemia-reperfusion injury in the heart.

16. Further, the inventors of the present invention have authored two articles unpublished but in press, as listed below:

⑤ Taniyama et al, Therapeutic Angiogenesis Induced by Human Hepatocyte Growth Factor Gene in Rat and Rabbit Hind Limb Ischemia Model.

This article reports that the gene therapy using the HGF gene was effective for hind limb ischemia.

⑥ Aoki et al., Angiogenesis Induced by Hepatocyte Growth Factor in Non-infarcted Myocardium and Infarcted Myocardium.

This article reports that the gene therapy using the HGF gene was effective for infarcted myocardium on the same procedure as that used in Test Example 8 of the present specification.

17. All the copies of Articles ① - ⑥ as listed above are attached hereto, and the copies of Articles ① and ② were also attached to the Amendment filed on June 30, 1999 in the above-identified application.

Rejection under 35 U.S.C. § 112, First Paragraph in the Final Office Action

18. I have noted that the Examiner has stated in the Final Office Action at page 3, line 17 to page 4, line 2 as follows:

-- This evidence is deemed to provide sufficient support for the enablement of a pharmaceutical composition comprising a liposome fused to Sendai virus and comprising a plasmid expression vector containing the HGF gene and a method of treating patients with the pharmaceutical composition by direct administration to the target site.--

Thus, the Examiner has admitted that the evidences, Articles ① and ② as stated hereinabove, sufficiently support the pharmaceutical composition for use as a method of treatment when directly administered to the target site. With regard to the direct administration to the target site, I would like to point out that Articles ④, ⑤ and ⑥, in addition to Articles ① and ②, further sufficiently support the direct administration to the target site.

19. The direct administration includes the embodiment wherein the pharmaceutical composition containing the HGF gene is directly administered to an injured tissue. Such an embodiment is specifically described in Test Examples 8 and 9 of the specification.

Further, Articles ⑤ and ⑥ support the direct administration of the HGF gene to an injured tissue.

20. In the meantime, according to the present invention, the pharmaceutical composition containing the HGF gene may be preferably administered intramuscularly to the subject in need of the HGF protein. In this regard, Articles ②, ⑤ and ⑥ report that, when the HGF gene was intramuscularly administered to each of the animal models with liver cirrhosis, hind limb ischemia and infarcted myocardium, the HGF gene was effective for those diseases on those different organs. These evidences fully support the effective intramuscular administration of the HGF gene, together with the statements "The medicament may be administered ····· intramuscularly" in the specification at page 13, the last line to page 14, line 2 as well as Test Example 8 of the specification wherein the HGF gene was administered to the heart muscle of the rat.

21. In view of the foregoing, I believe that the above-identified application should enable a person skilled in the art to practice the direct administration of the HGF gene to the target site or injured tissue

and the intramuscular administration of the HGF gene as well.

22. I have noted that, in the Final Office Action dated August 11, 1999, the Examiner has accepted only the Sendai virus-fused liposome (HVJ-liposome) encapsulating the plasmid vector containing the HGF gene as the dosage form in the present invention. However, I entirely disagree with the Examiner's position, and the Examiner's position should be unfounded for the reasons set forth below.

23. Since the Test Examples of the specification have demonstrated and confirmed that the gene therapy using the HGF gene is effective for various diseases, we could have highly expected, in view of the extremely potent activity of the HGF protein per se as a cell growth stimulation factor, that any dosage forms of the HGF gene according to a viral expression vector method, a naked-DNA method using an expression plasmid containig the HGF gene, and the like would effective as the gene therapy similarly to the HVJ-liposome method used at the Test Examples of the specification. In this regard, the specification states at page 9, lines 5 - 8 and page 12, lines 18 - 24, respectively, as follows:

-- ····· the HGF gene may be used in the form of a viral vector having the HGF gene as described hereinafter, or in the form of an appropriate expression vector having the HGF gene. --

-- For introduction of the HGF gene into cells, conventional methods are employed, which are roughly classified into introduction via viral vectors and other strategies. Both methods are available for the preparation of the medicament of the present invention. --

24. Indeed, the above statements in the specification are supported by the experimental data in Articles ③ and ⑤ as listed hereinabove. More specifically, Articles ③ reports that the transfection of the HGF gene to the animal model with injured lung according to Adenoviral vector containing the HGF gene was effective as the gene therapy using the HGF gene. Also, Article ⑤ reports that the intramuscular injection of "naked" human HGF plasmid into rat hindlimb ischemia model resulted in a significant increase in blood flow as stated at page 3 , lines 8 - 10. The naked-DNA method using the "naked" human HGF plasmid is a simple and

convenient method wherein a non-viral expression vector containing the HGF gene is directly transfected to the subject in need of the HGF protein without the use of viral particles, liposomes and the like. Therefore, according to even the naked-DNA method, the gene therapy using the HGF gene is also effective in the present invention.

25. As is clear from the above statements, the use of any dosage forms of the HGF gene is effective in the present invention, which I believe is sufficiently supported by the descriptions in the specification of the above-identified application and has been confirmed by the Articles published after the effective filing date of the present application.

26. In the meantime, I have noted that, in the Final Office Action, the Examiner has pointed out based on the teachings of Kohn et al. that "the actual gene therapy treatment was a disappointment, most likely due to the inefficient gene delivery system." As pointed out by the Examiner, the gene therapy technique might not yet, in general, been established in the art. However, I believe that this general knowledge in the art should not be applied to the gene therapy using the

HGF gene. As discussed in detail hereinabove, it has been well acknowldged in the art that the HGF protein per se functions via the c-Met receptor to be effective for various diseases. Further, the Japanese News Papers attached hereto has announced that the HGF protein have attracted public attention due to the extremely potent activity for regenerating liver even if cut off the half of the liver. Also, Articile ⓪, Francesco Galimi et al., STEM CELLS, 1993; 11: 22 - 30, of which copy is attached hereto, states at page 22, the right column, lines 11 - 15, as follows:

> -- HGF is considered to be the major mediator of liver regeneration in vivo; it is a powerful mitogen for several cell types, including hepatocytes, kindney tubular epithelium, keratinocytes, endothelial cells and melanocytes. --

Thus, the HGF protein is well known in the art to be an extremely potent cell growth stimulation factor. In view of the unique characteristic property of the HGF protein, since the Test Examples of the specification have demonstrated that the HGF gene is effective as the gene therapy, I have had the assurance that any dosage forms

of the HGF gene would be used as the gene therapy. Indeed, my this assurance has been confirmed by the experimental data in the Articles published after the effective filing date of the present application.

Rejection under 35 U.S.C. § 103 (a) in the Final Office Action

27. I have noted that, in the Office Action, the Examiner has considered that the present invention using the HVJ-liposome (Sendai virus-fused liposome) is obvious over Thierry et al. or Isner each in view of Morishita te al. However, I believe that the present invention using any dosage forms in addition to the HVJ-liposome should have inventive step over any cited prior art references, for the reasons as set forth in detail below.

(1) Treatment method using HVJ-liposome containing HGF gene

28. Thierry et al. does not describe a HVJ-liposome but merely teaches the method for encapsulating high molecular weight nucleic acids in liposomes. Noticeably, Thierry et al. describes nothing

whatsoever of the specific treatment of diseases. Morishita et al. does not describe anything of the HGF gene and further provide nothing whatsoever of the specific treatment of diseases, although Morishita et al. teaches a HVJ-liposome.

29. Isner teaches the treatment of arterial diseases, as pointed out by the Examiner in the Final Office Action. However, Isner does not specifically describe the effect of HGF gene, although Isner provides the pharmacological data for a VEGF gene. Even though the VEGF gene was effective as taught in Isner, a person skilled in the art could not have predicted whether the HGF gene might be similarly effective, for the reasons as given below.

30. At the outset, I would like to emphasize that the HGF protein is definitely different as the substance from the VEGF protein. More specifically, the HGF protein has a molecular weight of about 100 kDa which is larger by twice than that of the VEGF protein. Further, the HGF protein is initially produced in vivo as a single chain protein, and then cleaved to the double chain protein to exhibit its biological activity. In this regard, the HGF protein is entirely contrasted

to the VEGF protein in the mechanism for exerting the biological activity. Therefore, even if the VEGF gene was effectively transfected, it could not have been predicted whether the HGF gene after transfected would be correctly expressed and processed to the double chain protein for exerting its biological activity. Furthermore, the receptor of the HGF protein is c-Met, which is different from the receptor of the VEGF protein, resulting in the different activity on vascular cells. Indeed, the VEGF protein has inclusively three activities, angiogenesis activity, vascular permeation activity and vasodepressor activity, which are important to the biological activity of the VEGF protein. In contrast, the HGF protein has substantially only angiogenesis activity, and has no vascular permeation activity. Thus, in view of these differences between the HGF and VEGF proteins, a person skilled in the art could not have predicted whether the HGF gene might be effective as the gene therapy. In this regard, Isner does not teach anything. Isner states from the column 3, line 40, as follows:

-- "Such proteins include, for example, acidic and basic fibroblast growth factors (aFGF and bFGF), vascular endotherial growth factor

(VEGF), ····· hepatocyte growth factor (HGF) ···· --

Thus, Isner exemplifies all the possible angiogenesis factors, but the bFGF gene encoding the exemplified bFGF protein would be suspected to be ineffective as a gene therapy due to no signal sequence, although the bFGF protein has a potent angiogenesis activity. Under these circumstances, even though a protein was known to have a potent angiogenesis activity, a person skilled in the art could not have predicted whether the protein gene might be effective as a gene therapy, before conducting experiments to confirm the effectiveness as the gene therapy. Furthermore, Isner describes the different procedure wherein a catheter having hydrophilic polymers containing the VEGF gene attached to the end thereof was inserted to arterial vessel whereby the VEGF protein expressed was acted on the arterial vessel. Thus, the procedure of Isner was quite unique and complicated and therefore entirely different from the administration method of the present invention wherein the HGF gene is directly administered to the injured tissue or intramuscularly administered.

31. Accordingly, the views of the Examiner should be unjustified that "applicants' showing that HGF liposomes enable efficient transfection and expression of the HGF gene in arterial cells is exactly what one of ordinary skill in the art would expect following a reading of the cited references." In view of the foregoing discussions, I believe that the treatment method using the HVJ-liposome containing the HGF gene according the present invention is not obvious over Thierry et al., Isner and Morishita et al., singly or in combination therewith. Furthermore, I also believe that the treatment method using any dosage forms of the HGF gene is not obvious over Thierry et al., Isner and Morishita et al., singly or in combination therewith.

(2) Pharmaceutical composition comprising HVJ-liposome containing HGF gene

32. The pharmaceutical composition comprising HVJ-liposome containing the HGF gene according to the present invention indicates a pharmaceutical composition actually exhibiting a therapeutic effect for diseases. On the other hand, Thierry et al. describes nothing of a HVJ-liposome, although Thierry et al. teaches a liposome. Thierry et al. teaches no more than the method for

encapsulating high molecular weight nucleic acids in liposomes. Thierry et al. describes neither the in vivo effect nor the therapeutical effect of the liposomes encapsulating the nucleic acids.

33. Isner et al. does not teach anything of a HVJ-liposome actually exhibiting the pharmacological effect. Such a liposome would not be obvious to a person skilled in the art from the teaching of Isner et al. Morishita et al. does not teach anything whatsoever of the HGF gene, although Morisihita et al. describes a pharmaceutical composition comprising a HVJ-liposome. Morishita et al. merely describes a gene encoding ACE (angiotensin converting enzyme) irrelevant to the HGF gene. Furthermore, Morishita et al. teaches no more than that the ACE gene was transfected ex vivo to the cultured cells of vessel enucleated from rat and that the expression of the ACE gene was confirmed by immunostaining the cells. Morishita et al. does not teach anything of whether the ACE enzyme expressed in vivo would actually exhibit its enzymatic activity and therapeutical effect.

34. On the other hand, the HGF protein definitely differs from ACE enzyme in that the HGF

protein is a physiologically active protein having a complicated three-dimensional structure and requires various biological steps including the processing of the single chain protein to the double chain protein for exerting its biological functions. For the HGF gene encoding such a complicated protein, we have, for the first time, confirmed the therapeutical effect of the HGF gene when transfected to cells. Even though Morishita et al. teaches the transfection of the ACE gene into the cultured vascular cells, a person skilled in the art could not have easily arrived the pharmaceutical composition comprising the HVJ-liposome containing the HGF gene actually exhibiting the therapeutical effect, as achieved by the present invention.

35. In view of the foregoing discussions, I believe that the pharmaceutical composition comprising the HVJ-liposome containing the HGF gene according to the present invention is not obvious over any of the cited references singly or in combination therewith.

The undersigned declarant declares further that all statements made herein of his own knowledge are true and that all statements made on information and

belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Signed this 20th day of January, 2000.

Ryuichi MORISHITA

Attachment #1

American Heart Association℠
Fighting Heart Disease and Stroke

USAN 09/029,41
Assignee: Sumitomo
Inventor: Morishi
Filed: June 9, 19

Supplement to Circulation

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Abstracts From the 70th Scientific Sessions
Orange County Convention Center
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Abstracts From the 70th Scientific Sessions

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charges.

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Sulfonylureas are not associated with increased acute myocardial infarction mortality

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Despite early epidemiological data and more recent experimental data suggesting increased cardiovascular mortality with sulfonylureas, the majority of patients with non-insulin-dependent diabetes mellitus (DM) are treated with these agents. We examined the survival of 64,171 elderly acute myocardial infarction (MI) patients with DM (mean age 75, female 53%, non-white 15%) according to therapy on admission as part of the Health Care Financing Administration's Cooperative Cardiovascular Project. Patients taking sulfonylureas were less likely to present with shock, and more likely to survive hospitalization compared to patient treated with insulin, or diet therapy alone. After adjusting for age, gender, race, infarct location, Killip class, history of MI or congestive heart failure, cerebral and peripheral vascular disease, renal insufficiency, and smoking, patients treated with sulfonylurea agents continued to have the highest in-hospital survival (P<0.002) compared to diet therapy or insulin therapy. While unmeasured differences in illness severity may have accounted for some portion of the differences in survival, this observational study suggests that sulfonylureas may be safely used to treat elderly patients with coronary artery disease and diabetes mellitus.

	Number	Age (mean)	Female %	Anterior MI %
Sulfonylurea	25,035	75.5	48.9	46.3
Insulin	18,935	74.5	58.0	44.1
Diet	17,851	76.1	51.5	45.2
No DM	143,248	75.5	46.4	44.8

	Shock %	Renal insuf. %	Cardiac arrest %	Hospital death %
Sulfonylurea	7.4	11.5	11.3	14.7
Insulin	7.8	16.6	11.7	16.1
Diet	9.0	16.4	13.2	17.7
No DM	7.0	9.2	11.3	13.8

Cardio-Thoracic And Vascular Surgery:

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Abstracts 3459 - 3471

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In Vivo Gene Transfection of Hepatocyte Growth Factor Attenuates Ischemia-Reperfusion Injury In The Heart : Evidence for A Role of HGF in Endogenous Myocardial Protection

Hideki Ueda, Yoshiki Sawa, Youichi Kawahira, Koji Kagisaki, Kunio Matsumoto, Toshikazu Nakamura, Hikaru Matsuda, Osaka Medical College, Osaka Japan

Hepatocyte growth factor(HGF) plays a role as mitogen, motogen and morphogen mainly for epithelial cells and it also promotes cell survival in liver and central nervous system. Though c-met, a high affinity HGF receptor, is constitutionally expressed in the heart, especially in coronary endothelial cells, a role of HGF in the heart is undetermined exclusive of promoting angiogenesis after myocardial ischemia. In this study, we try to assess the chronological kinetics of endogenous HGF and c-met in the heart after myocardial ischemia and clarify whether HGF may attenuate ischemia reperfusion (I-R) injury in the heart using in vivo gene transfection of human HGF into the heart with HVJ liposome method. <Kinetics of endogenous HGF> Total RNA was extracted from Wistar rat (8weeks after birth) hearts from 3hours to 14days after myocardial ischemia by left coronary artery ligation. Stronger band of RT-PCR products from HGF mRNA was detected at 3days after myocardial ischemia and that of c-met mRNA was also detected at 24 hours after ischemia.<Roles of HGF> After isolation of Wistar male rat (n=12, 200-250g) hearts, these hearts were transfected HVZ liposome with or without human HGF cDNA (HGF group(n=6) vs. control group(n=6)). They were transplanted in the abdomen of recipient rats of the same strain. At 4 days after transplantation, these hearts were isolated and perfused with Krebs-Hensaleit solution. Left ventricular developed pressure (LVDP), heart rate (HR) and coronary flow (CF) were measured before and after 30 min. of warm ischemia (37° C) followed by 30 min. of reperfusion. To clarify evidence for gene transfection, we confirmed RT-PCR products from human HGF mRNA using primers set to little anneal rat HGF mRNA. Recovery ratio of LVDP (H vs. C, 113±36 vs. 76±9%) were significantly higher in the HGF group than in control group (p=0.03). Recovery ratio of HR and CF in both groups did not show significant difference. Creatine kinase (CK) leakage in the coronary effluent during 5 min. after reperfusion (H vs. C, 0±0 vs. 71±47 mIU/5min.) were significantly lower in the HGF group than in control group (p=0.002). These results suggested that myocardial ischemia induced endogenous HGF and c-met in the rat heart and in vivo gene transfection of HGF with HVJ liposome method attenuates I-R inj...

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Evidence for Interleukin-6 and Intercellular Adhesion Molecule-1 Induction in the Canine Myocardium Following Cardiopulmonary Bypass

William J Dreyer, Texas Children's Hospital, Houston, TX; Merry L Lindsey, Peggy Jackson, Sharon C Phillips, Baylor College of Medicine, Houston, TX

A systemic inflammatory reaction occurs in response to cardiopulmonary bypass (CPB). Recent evidence suggests myocardial involvement in this response. Interleukin-6 (IL-6) is a pro-inflammatory cytokine known to induce cardiac myocyte intercellular adhesion molecule-1 (ICAM-1) production (thus supporting neutrophil-myocyte adhesion) which recently has been shown to have negative inotropic properties as well. IL-6 regulation after CPB is unknown. To examine IL-6 regulation further, dogs underwent 90 mins of hypothermic CPB (25-28° C)with 50 mins of cardioplegic arrest. After rewarming, dogs were reperfused open chest for either 2 hrs (n=4) or 6 hrs (n=4) at the end of which myocardial biopsies were obtained. An additional 4 dogs undergoing open thoracotomy without CPB served as time-matched controls. Northern blot analysis of myocardial mRNA showed a marked increase in IL-6 in 3/4 dogs at 3 hrs and 3/4 dogs at 6 hrs of reperfusion when compared to sham bypass controls. Northern blots of mRNA extracted from isolated neutrophils and mononuclear leukocytes obtained from blood samples prior to CPB, at the end of CPB, and at 3 hrs of reperfusion failed to demonstrate IL-6 induction. In situ hybridization studies of the biopsied myocardium confirmed cardiac myocytes as a primary source of IL-6 production after CPB. Results of northern blots for ICAM-1 performed on the same myocardial tissue as the IL-6 studies showed ICAM-1 induction which was highly concordant with the IL-6 results. Thus, ischemia/reperfusion during CPB, despite myocardial protection with cold cardioplegic arrest, appears to be a sufficient stimulus to induce myocardial IL-6 synthesis. Myocardial IL-6 production may play a pivotal role in the development of depressed myocardial function postoperatively.

3461

A Hybrid Artificial Lung wit IL-10 and ecNOS Genes Transfected Endothelial Cells Attenuates the Inflammatory Reactions Induced by CPB

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In cardiopulmonary bypass (CPB), the direct contact of the blood to the artificial lung (AL) evokes the inflammatory reactions (IR) through the complements and neutrophils (PMN) activation and the cytokine production resulting in post perfusion syndrome. For an attenuation of these reactions, hybrid AL in which vascular endothelial cells (EC) are cultured on the surface of hollow fibers may be an alternative. Recently, the inhibitory effects of nitric oxide (NO) on PMN and platelet activation and those of interleukin(IL)-10 on production of inflammatory cytokines such as IL-8, -6 and TNF-α are proposed in the pathogenesis of inflammation. In this study, we investigated the efficacy of the hybrid AL with ecNOS and IL-10 gene-transfected EC to attenuate IR during CPB. In vitro experiment: ecNOS and IL-10 gene were transfected using HVJ (Hemagglutinating Virus of Japan)-liposome method. The efficacy of the transfection was evaluated by measuring NO, IL-8 and IL-10 level in the supernatant from transfected EC after the stimulation of LPS or TNF-α. NO produced by ecNOS-transfected EC was significantly higher than that by control EC (284.5±54.0 vs 95.7±27.9 μM, p<0.01). IL-8 production by IL-10-transfected EC was significantly decreased than that by control EC (3.1±2.4 vs 62.1±1.3 ng/ml <0.001). IL-10 was detected only the transfected EC (5 pg/ml), while not in the control EC. In vivo experiment: partial CPB for an hour was established in rats to evaluate the serum level o NO and IL-8 just after CPB with our hybrid AL. Hybrid AL without EC (C group;n=3), coated with non-treated EC (E;n=3) or coated with ecNOS and IL-10-transfected EC (T;n=3)were used. At 4 min. of CPB, T group showed higher level of serum NO as compared to C and E group. Serum IL-8 level in the T group just after CPB was significantly lower than that in the C grou (1728±282 vs 4275±145 pg/ml, p=0.0151). These results suggest that hybrid AL with IL-10 an ecNOS genes transfected EC may attenuate the IR induced by CPB.

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Prolonged Hypothermic Circulatory Arrest Causes Neurologic Sequelae Despite Somatic Recovery in the Rat

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Objective: Integrated neurologic function, behavior and somatic recovery were studied in rats undergoing 5 to 80 minutes (min) of deep hypothermic circulatory arrest (HCA). Metho A miniature extracorporeal circulation system (ECC) consisting of an oxygenator and l exchanger, primed with 6 ml of asanguinous solution, was connected to a closed-chest rat cannulae in the right atrium for venous drainage (arterial return (18 centigrades, when ECC stopped and cardioplegic solution delivered. After 5, 10, 20, 40 min (each n=5) and 80 (Group L, n=15) of HCA, the rat was reperfused, weaned from ECC and followed by behav scoring, passive avoidance tasks and cardiopulmonary exercise testing until sacrifice morphologic studies. Results: Every rat resumed weight gain in the first week and rega exercise capacity by the second week after HCA. Only Group L rats showed behav abnormalities such as stereotypy and incomplete righting reflex, which disappeared in the f week. Learning ability was preserved in all except Group L rats (n=10), who failed to acquire memory to avoid electric stimuli up to 3 months after HCA, when pyramidal cells were l replaced by astroglia in CA1 sector of hyppocampus and cerebral cortex. Nonetheless memory established before HCA was preserved and allowed Group L animals (n=5) to

In Vivo Gene Transfection of Hepatocyte Growth Factor Attenuates Ischemia-Reperfusion Injury In The Heart : Evidence for A Role of HGF in Endogenous Myocardial Protection

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Hepatocyte growth factor(HGF) plays a role as mitogen, motogen and morphogen mainly for epithelial cells and it also promotes cell survival in liver and central nervous system. Though c-met, a high affinity HGF receptor, is constitutionally expressed in the heart, especially in coronary endothelial cells, a role of HGF in the heart is undetermined exclusive of promoting angiogenesis after myocardial ischemia. In this study, we try to assess the chronological kinetics of endogenous HGF and c-met in the heart after myocardial ischemia and clarify whether HGF may attenuate ischemia reperfusion (I-R) injury in the heart using in vivo gene transfection of human HGF into the heart with HVJ liposome method. <Kinetics of endogenous HGF> Total RNA was extracted from Wistar rat (8weeks after birth) hearts from 3hours to 14days after myocardial ischemia by left coronary artery ligation. Stronger band of RT-PCR products from HGF mRNA was detected at 3days after myocardial ischemia and that of c-met mRNA was also detected at 24 hours after ischemia.<Roles of HGF> After isolation of Wistar male rat (n=12, 200-250g) hearts, these hearts were transfected HVZ liposome with or without human HGF cDNA (HGF group(n=6) vs. control group(n=6)). They were transplanted in the abdomen of recipient rats of the same strain. At 4 days after transplantation, these hearts were isolated and perfused with Krebs-Henseleit solution. Left ventricular developed pressure (LVDP), heart rate (HR) and coronary flow (CF) were measured before and after 30 min. of warm ischemia (37° C) followed by 30 min. of reperfusion. To clarify evidence for gene transfection, we confirmed RT-PCR products from human HGF mRNA using primers set to little anneal rat HGF mRNA. Recovery ratio of LVDP (H vs. C, 113±36 vs. 76±9%) were significantly higher in the HGF group than in control group (p=0.03). Recovery ratio of HR and CF in both groups did not show significant difference. Creatine kinase (CK) leakage in the coronary effluent during 5 min. after reperfusion (H vs. C, 0±0 vs, 71±47 mIU/5min.) were significantly lower in the HGF group than in control group (p=0.002). These results suggested that myocardial ischemia induced endogenous HGF and c-met in the rat heart and in vivo gene transfection of HGF with HVJ liposome method attenuates I-R injury in it.

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Hepatocyte growth factor gene therapy of liver cirrhosis in rats

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Liver cirrhosis is the irreversible end result of fibrous scarring and hepatocellular regeneration, characterized by diffuse disorganization of the normal hepatic structure of regenerative nodules and fibrotic tissue[1]. It is associated with prominent morbidity and mortality, and is induced by many factors, including chronic hepatitis virus infections, alcohol drinking and drug abuse. Hepatocyte growth factor (HGF), originally identified and cloned as a potent mitogen for hepatocytes[2-5], shows mitogenic, motogenic and morphogenic activities for a wide variety of cells[6-9]. Moreover, HGF plays an essential part in the development and regeneration of the liver[4,7,10], and shows anti-apoptotic activity in hepatocytes[11]. In a rat model of lethal liver cirrhosis produced by dimethylnitrosamine administrations, repeated transfections of the human HGF gene into skeletal muscles induced a high plasma level of human as well as enodogenous rat HGF, and tyrosine phosphorylation of the c-Met/HGF receptor. Transduction with the HGF gene also suppressed the increase of transforming growth factor-β1 (TGF-β1), which plays an essential part in the progression of liver cirrhosis, inhibited fibrogenesis and hepatocyte apoptosis, and produced the complete resolution of fibrosis in the cirrhotic liver, thereby improving the survival rate of rats with this severe illness. Thus, HGF gene therapy may be potentially useful for the treatment of patients with liver cirrhosis, which is otherwise fatal and untreatable by conventional therapy.

Dimethylnitrosamine (DMN) was administered intraperitoneally to rats for 3 consecutive days each week (Fig. 1*a*). After the fourth weekly administration of DMN, histological examination of liver specimens showed the collapse of parenchymal cells and the formation of regenerative nodules separated by fibrous septa. Reticulin fibers spread radially throughout the liver. The formation of thin fibrotic septa joining the central areas was observed, and a micronodular pattern of the parenchyma was evident in all rats (Fig. 1*b*), similar to the characteristic pathological changes found in liver cirrhosis in humans[12,13]. This illness was progressive and fatal to the rats, which all died after the seventh weekly DMN administration (Fig. 1*f*). To treat animals with this advanced disease, we established a simple and safe *in vivo* transfection procedure of repeatedly transducing skeletal muscles with the HGF gene using liposomes containing the hemagglutinating virus of Japan[14-16] (HVJ liposomes). Beginning after the fourth weekly DMN administration (when there is serious liver cirrhosis), rats were injected in their skeletal muscles once a week either with HVJ liposomes containing 20 mg or 40 mg of human HGF expression vector (HGF-HVJ liposomes), or with phosphate-buffered saline (PBS control) (Fig. 1*a*). After a single injection of HGF-HVJ liposomes, a substantial amount of human HGF was detected by enzyme-linked immunosorbent assay (ELISA) in the plasma of rats for more than a week (Fig. 1*c*). Inoculation of more HGF-HVJ liposomes 1 week later further increased the plasma level of human HGF (Fig. 1*c*). Similar results were obtained by ELISA using homogenates of muscle tissues injected with HGF-HVJ liposomes (data not shown). Plasma levels of endogenous HGF were assessed by ELISA specific for rat HGF. They were increased 1 week after the first DMN administration, and then gradually decreased to normal levels despite subsequent DMN administrations (Fig. 1*c*). When these rats were transfected with HGF-HVJ liposomes, the level of endogenous HGF was not suppressed by transduction with the human HGF gene, but it was further increased (Fig. 1*d*). Transfection of the human HGF gene in rat smooth muscle cells increases the production of endogenous rat HGF *in vitro*[17]. Our observation supports the idea that transduction of skeletal muscle with the human HGF gene boosts endogenous rat HGF *in vivo* as well.

The receptor for HGF is a tyrosine kinase receptor encoded by c-*met*. The membrane-spanning β-chain of the c-Met/HGF receptor contains an intracellular tyrosine kinase domain[18]. c-Met protein was expressed in normal rat liver at a low level, as demonstrated by western blotting (Fig. 1*e*, top, lane 1). The level of c-Met protein in the liver was transiently increased (Fig. 1*e*, top, lanes 2 and 3) and then decreased by DMN administration in PBS control rats (Fig. 1*e*, top, lane 4). In contrast, c-Met protein levels increased considerably in rats treated with HGF-HVJ liposomes, and stayed at this high level even after the repeated DMN administration (Fig. 1*e*, top, lane 5). The biological activities of HGF are produced by the phosphorylation of tyrosine residue in c-Met. Tyrosine phosphorylation of c-Met was not seen in normal liver (Fig. 1*e*, bottom, lane 1) or in livers from

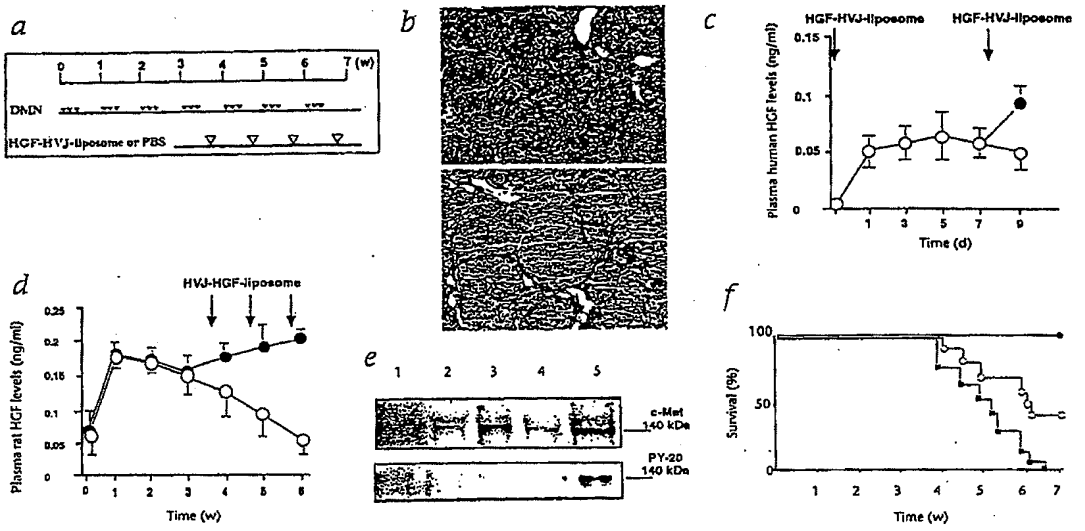

Fig. 1 *a*, Schedule of DMN administrations and HGF-HVJ liposome or PBS injections. w, weeks. *b*, Liver specimens from rats treated with DMN for 4 weeks, stained with Azan-Mallory staining (upper) or silver impregnation (lower). Original magnification, ×100. *c*, Plasma levels of human HGF induced by HGF-HVJ liposome injection in DMN-treated rats. ○, HGF serum level of rats that received a single gene injection ($n = 7$); ●, HGF plasma level of rats that received a second HGF-HVJ liposome injection ($n = 5$). Downward arrows indicate time of HGF treatment. The second injection enhanced the human HGF level. All values are presented as mean ± standard deviation. *d*, Plasma levels of endogenous HGF (rat HGF) in DMN-treated rats. ○, HGF level of control rats without HGF-HVJ liposome treatment ($n = 8$); ●, HGF level of rats that received repeated HGF-HVJ liposome injections ($n = 7$). Downward arrows indicate time of HGF treatment. All values are presented as mean ± standard deviation. *e*, Western immunoblot analysis of expression of c-Met (upper) and tyrosine phosphorylation of c-Met (PY-20; lower) in liver homogenates. Normal rat liver (lane 1); livers from rats treated with DMN for 2 weeks (lane 2), 4 weeks (lane 3), and 6 weeks (lane 4); liver from rat treated with DMN for 6 weeks and HGF-HVJ liposomes for the last 3 weeks (lane 5). *f*, Survival of rats in a control group that were injected with PBS (■; $n = 13$), or rats that received repeated injection of HGF-HVJ liposomes containing 20 mg (○; $n = 13$) or 40 mg of HGF DNA (●; $n = 9$). Life-table analyses are presented as a Kaplan-Meyer plot.

PBS control rats given DMN (Fig. 1*e*, bottom, lanes 2–4). In contrast, strong tyrosine phosphorylation of c-Met was observed in the livers of rats given repeated injections of HGF-HVJ liposomes, despite DMN administration (Fig. 1*e*, bottom, lane 5). These results indicate that the introduction of the human HGF gene induced human HGF and endogenous rat HGF, and strongly activated the c-Met receptor in the cirrhotic liver.

Overproduction of transforming growth factor-β (ref. 1; TGF-β1) is a chief cause of tissue fibrosis in various organs[19, 20]. TGF-β1 induces the phenotypic transition of hepatic stellate cells to proliferating myofibroblast-like cells, which enhances the production of extracellular matrix components[21, 22] and attenuates the degradation of the extracellular matrix proteins[22, 23]. TGF-β1 transgenic mice that produce high plasma levels of TGF-β1 develop liver fibrosis[22]. TGF-β1 is also a potent growth inhibitor of epithelial and endothelial cells, including hepatocytes[23]. Furthermore, TGF-β1 induces apoptotic cell death in hepatocytes[22, 24]. We therefore examined expression of TGF-β1 in cirrhotic rat liver. Immunohistochemical analysis demonstrated the presence of TGF-β1-positive cells widely distributed in the livers of DMN-treated rats (Fig. 2*a*, left). HGF-HVJ liposome injections substantially decreased the number of TGF-β1-positive cells (Fig. 2*a*, right). Consistent with this, the level of TGF-β1 mRNA expression was reduced by the treatment with HGF-HVJ liposomes (Fig. 2*e*, lanes 5 and 6), in distinct contrast to the high expression of TGF-β1 in PBS control rats (Fig. 2*e*, lanes 2–4).

In the cirrhotic liver, hepatic stellate cells that are positive for desmin are increased in the fibrotic regions, and many of them are transformed into myofiblolast-like cells that specifically express α-smooth muscle actin[25] (α-SMA). We examined the expression of α-SMA in rat liver by immunohistochemistry and western blotting, and found that it correlated well with the expression of TGF-β1. The number of α-SMA-positive cells was considerably increased in the cirrhotic livers of PBS control rats (Fig. 2*b*, left), whereas it was notably inhibited in the livers of rats transfected with the HGF gene (Fig. 2*b*, right). Similar results were obtained by western analysis of α-SMA protein in the liver (Fig. 2*f*). Thus, transduction of rat skeletal muscles with human HGF gene suppressed the expression of TGF-β1, and inhibited transition from hepatic stellate cells to myofibroblast-like cells in the cirrhotic livers of rats. Consistent with these findings, fibrous connective tissue components in Glisson's sheath and pseudolobule formations found in the livers of DMN-treated rats (Fig. 3*a*) were inhibited by repeated HGF-HVJ-liposome treatments (Fig. 3*b*). Formation of fibrotic septa and thickened reticulin fibers joining central areas were observed (Fig. 3*a*). In rats injected repeatedly with HGF-HVJ liposomes,

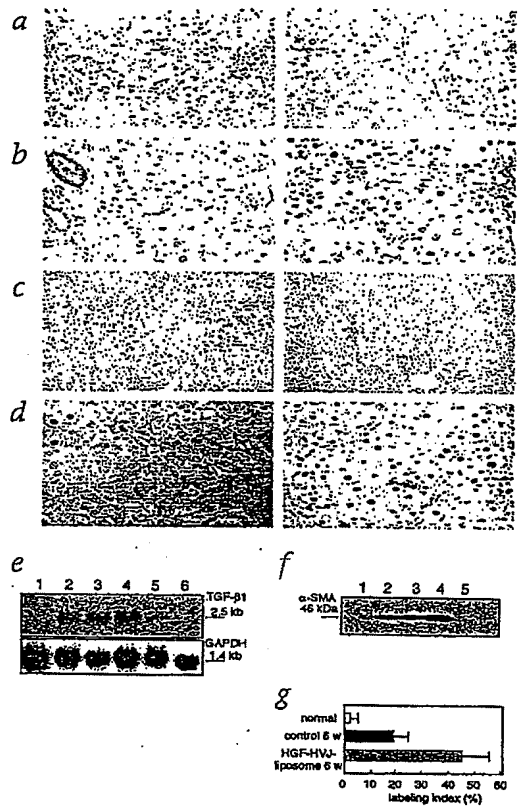

Fig. 2 a–d, Livers were obtained from rats 6 weeks after DMN administration began. Left panels, control rats injected with PBS for 3 weeks; right panels, rats injected with HGF-HVJ liposomes containing 40 mg of HGF DNA for 3 weeks. *a* and *b*, Immunohistochemical staining of liver sections using anti-TGF-β1 antibody (a) or anti-α-SMA antibody (b). Original magnification, ×200. *c*, Apoptosis of hepatocytes. TUNEL was used to detect apoptotic cells in the livers. Original magnification, ×100. *d*, PCNA staining. Mitosis in hepatocyte was assessed by immunohistochemistry using anti-PCNA antibody. Original magnification, ×200. *e*, Northern blot analysis of TGF-β1 (upper) and glyceraldehyde-3-phosphate dehydrogenase (lower) mRNA. Total liver RNAs were derived from a normal rat (lane 1); rats treated with DMN for 2 weeks (lane 2), 4 weeks (lane 3) or 6 weeks (lane 4); or rats that received repeated injections of HGF-HVJ liposomes containing 20 mg (lane 5) or 40 mg (lane 6) of HGF DNA for 3 weeks. *f*, Western blot analysis of α-SMA. Liver protein was prepared from normal rat (lane 1); rats treated with DMN for 2 weeks (lane 2), 4 weeks (lane 3) or 6 weeks without HGF-HVJ-liposome treatment (lane 4) or with HGF-HVJ-liposome treatment for 3 weeks (40 mg of HGF DNA) (lane 5). *g*, Labeling index of PCNA-positive hepatocytes. Open bar, normal rats; filled bar, rats treated with DMN for 6 weeks without HGF-HVJ-liposome treatment; shaded bar, rats treated with DMN and injected with HGF-HVJ liposomes for 6 weeks. Values are presented as mean ± standard deviation.

fibrosis in both the periportal and centrilobular liver diminished and deformation of the liver acinus decreased (Fig. 3*b*). These histological improvements were found to be consistent in the treated rats, even at later time points (Fig. 3*c*). Quantitative analysis of fibrosis by image analysis techniques[26] showed a more than 70% reduction of fibrosis after HGF-HVJ-liposome treatment (Fig. 3*d*).

DMN induces apoptosis of hepatocytes[13]. We therefore determined whether introduction of the HGF gene inhibited apoptosis of hepatocytes. Apoptotic cells were assessed by TUNEL, which detects fragmented DNA *in situ* (Fig. 2*c*). Apoptotic cells were found in the cirrhotic livers of DMN-treated rats (Fig. 2*c*, left). HGF gene therapy prevented this DMN-induced hepatocyte apoptosis (Fig. 2*c*, right). Because HGF is a potent hepatocyte mitogen, we also assessed the presence of mitotic hepatocytes by immunohistochemical staining, using antibody specific for proliferative cell nuclear antigen (PCNA) (Fig. 2*d*). The number of PCNA-positive hepatocytes was higher in the livers of rats treated with HGF-HVJ liposomes (Fig. 2*d*, right) than in the livers of the PBS control rats (Fig. 2*d*, left). The labeling index showed that 47% of hepatocytes were in the mitotic cycle in HGF-transfected rats (Fig. 2*g*).

We also determined the length of survival of rats treated with HGF-HVJ liposomes (Fig. 1*f*). All placebo-treated rats died of progressive liver cirrhosis within 45 days after DMN administration began (survival 25–45 days; median survival, 34 days; $n = 13$; Fig. 1*f*). In contrast, transfected rats (20 mg/rat) survived significantly longer than untreated control rats, and 6 of 13 rats survived more than 50 days (survival, 28 to more than 50 days; median survival, 43 days; $n = 13$) ($P < 0.01$, compared with untreated rats, by log rank analysis of the Kaplan-Meier curves). Transfection of rats with 40 mg of HGF DNA rescued all rats from fatal liver cirrhosis, and they were free of liver cirrhosis when they were removed from the study 50 days after DMN administration began ($n = 9$; $P < 0.001$, compared with untreated rats).

Here we have demonstrated that repeated transduction of skeletal muscles with the HGF gene produced complete resolution of fibrosis in the cirrhotic liver, inhibited hepatocyte apoptosis and stimulated hepatocyte mitosis, resulting in the survival of rats with an otherwise lethal illness. Although the molecular mechanisms of hepatic fibrosis have not been fully described, overexpression of TGF-β1 may play a pivotal part in the progression of fibosis[19–22]. Introduction of the HGF gene suppressed the expression of TGF-β induced by DMN. Thus, HGF gene therapy may have improved the liver cirrhosis by inhibiting TGF-β expression. Although tumozigenicity has been reported in transgenic mice overexpressing HGF, its expression was 5,000% higher in these mice than in normal mice[27]. We did not observe any tumor formation in mice treated with HGF gene therapy. In addition, transgenic mice that express HGF at levels similar to those in this experiment (200–300% higher than that of normal controls) inhibit the development of neoplastic tumors[28]. Repeated *in vivo* transfection using HVJ liposomes is simple and safe, and can be done without substantial inflammation or activation of the cellular and humoral immune system[16]. Therefore, transduction of skeletal muscles with the HGF gene as presented here may eventually be translated into a useful clinical regimen of gene therapy for the treatment of patients with progressive liver cirrhosis.

Methods

Animals. Sprague-Dawley rats 4–5 weeks old and 120–130 g in body weight were used. To produce liver cirrhosis, 1% DMN dissolved in saline

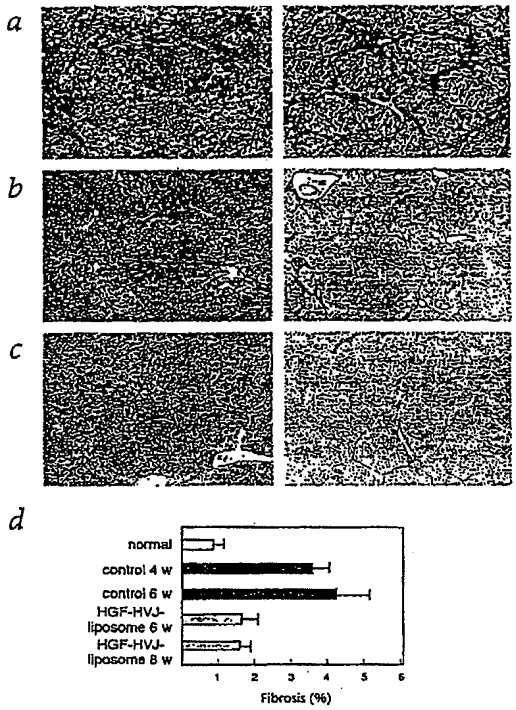

Fig. 3 a–c, Liver sections of rats after DMN administration. Left panels, Azan-Mallory staining; right panels, silver impregnation staining. *a*, Liver sections of control rats at 6 weeks after DMN administration began. *b* and *c*, Liver sections of rats injected with HGF-HVJ liposomes containing 40 mg of HGF DNA, at 6 weeks (b) or 8 weeks (c) after DMN administration began. *d*, Assessment of fibrosis using image analysis techniques, calculating the ratio of connective tissue to the whole area of the liver from rats treated with PBS or with repeated injection of HGF-HVJ liposomes . Values are presented as mean ± standard deviation. normal, normal rat; control 4 w, rats treated with DMN for 4 weeks without HGF-HVJ-liposome treatment; HGF-HVJ-liposome 6 w, rats treated with DMN and injected with HGF-HVJ-liposome for 6 weeks; HGF-HVJ-liposome 8 w, rats treated with DMN and injected with HGF-HVJ-liposome for 8 weeks.

was given intraperitoneally at 1 ml per kg body weight for 3 consecutive days per week for 4–6 weeks as described[17].

The expression vector and the preparation of HVJ-liposome complexes. Human HGF cDNA (2.2 kb) was inserted into the *Eco*RI and *Not*I sites of the pUC-SRa expression vector plasmid. In this plasmid, transcription of the HGF cDNA is under the control of the SRa promoter[18]. HVJ-liposomes containing plasmid DNA and high-mobility group 1 were constituted as described[15].

Gene transfer into rat skeletal muscles and *in vivo* experimental design. Rats were anesthetized with diethyl-ether, and their gluteal muscles were exposed. The HVJ liposome solution (20 mg or 40 mg of encapsulated DNA of human HGF cDNA) or PBS was injected into the muscles once a week after the development of liver cirrhosis.

Northern blot analysis. RNA extraction and northern blot analysis were done as described[15]. Total RNA (20 mg) was separated by electrophoresis on a 1.5% agarose–formaldehyde denaturing gel and transferred to a nylon membrane. The membrane was hybridized with DNA probes labelled with ^{32}P-dCTP by random oligonucleotide priming (Boehringer). The rat TGF-β1 cDNA probe was a gift from E. Imai (Osaka University Medical School). The membranes were washed and exposed to X-ray films. For the control study, membranes were washed and rehybridized with glyceraldehyde-3-phosphate dehydrogenase probe.

Western and immunoprecipitation analysis. Protein was extracted from homogenized tissues, and western blotting was done using samples normalized for protein concentration. The membranes were probed with anti-rat c-Met antibody (Santa Cruz Biotechnology, Santa Cruz, California) or anti-α-smooth muscle actin antibody (DAKO Japan, Kyoto, Japan). For immunoprecipitation, lysates were incubated with the anti-rat c-Met antibody at 4 °C overnight and incubated with protein A-agarose beads (Pierce, Rockford, Illinois) to precipitate the antigen–antibody complexes. Samples (containing 20 mg of total protein each) were separated by SDS–PAGE on 10% acrylamide slab gels. After electrophoresis, the gel was transferred to polyvinylidine difluoride sheets, which were subsequently probed with anti-phosphotyrosine antibody (PY-20; ICN) or anti-rat c-Met antibody.

Histological and immunohistochemical examination. Paraffin-embedded sections were stained with hematoxylin-eosin, Azan-Mallory and silver impregnation. Fibrosis was assessed using image analysis techniques[24], on Azan-Mallory-stained histologic slides 4 mm in thickness, using a planimetric method on an Automatic Image Analysis System (Carl Zeiss, Oberkochem, Germany). For immunohistochemical staining, the sections were treated with anti-α-SMA antibody, anti-PCNA antibody (PC-10; Novocastra, Newcastle, England) or anti-TGF-β1 antibody (R&D Systems, Minneapolis, Minnesota). The PCNA labeling index was determined by counting more than 2,000 nuclei of hepatocytes in three different sections for each rat. To detect apoptosis in the liver sections, a modified TUNEL method[10] was applied using the ApopTag *In situ* Apoptosis Detection System (Oncor, Gaitherburg, Maryland).

ELISA for HGF. Human HGF in serum was measured by ELISA using anti-human-HGF monoclonal antibody, and rat HGF in serum was measured by ELISA using anti-rat-HGF monoclonal antibody (Institute of Immunology, Tokyo, Japan). The human HGF ELISA system specifically detects human HGF but not rat HGF, and vice versa.

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平成８年度研究報告書

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びまん性肺疾患分科会

分科会長　工 藤 翔 二

薬剤性肺傷害に対するHGFcDNA組込み Adenovirus vectorによる遺伝子治療

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はじめに

Hepatocyte growth factor（HGF）は，発見当初は肝細胞に特異性の高い増殖因子と考えられていた[1,2]．その受容体はc-Metと同定され，肝のみならず腎臓やその他の臓器に分布する事が明らかになった[3,4]．HGFはその後の研究で多様な生物活性を有し，組織器官の形成や再生に中心的な作用を有する事が示されている．HGFは間葉系の細胞で産生され，上皮系の細胞に作用する上皮－間葉相互作用のメディエーターであり，上皮細胞のアポトーシスを抑制し，増殖を促進することによって組織傷害に対する抑制効果・組織修復効果を示すと考えられている．我々はリコンビナントHGF投与により薬剤性肺傷害が抑制されることを示し，HGFが肝臓や腎臓[5,6]のみならず肺においても肺再生因子として作用することを報告している[7]．今回実験肺傷害マウスにおける高濃度・長期間のHGF持続発現を目的とし，ラットHGFcDNA組み込みAdenovirus vectorを用い遺伝子導入を試みた．

対象と方法

マウスはC57BL／6，Female，8-12週齢を，adenovirus vectorはrat HGF cDNAを組み込んだAdex1CAHHGF（癌研究会癌化学療法センター濱田洋文博士より提供）を用いた（Fig. 1）．このadenovirus vectorはadenovirus geneのEla，b及びE3に欠損を持ち感染細胞内において非増殖性である．またCAGプロモーターを持ち導入遺伝子の効率的な発現を得るようデザインされている．

マウスの背側皮下に埋め込んだ浸透圧性ミニポンプ（Model 2001，Alza社）を用いてBleomycin（BLM）100mg／kgを1週間持続皮下投与しマウスに肺傷害を惹起した．Bleomycin投与開始と同時にadenovirusvector：Adex1CAHHGF 6×10^8pfu／mouseを腹腔内投与した．

マウスをadenovirus vector・BLM投与群とBLM・生食投与群の2群に分けた．adenovirus vector，BLM投与開始後4週後にマウスを屠殺しadenovirus vectorによって遺伝子導入発現したratHGFによるBLMによる肺傷害の抑制効果を検討した．肺摘除後ホルマリン固定しelastica-Masson染色を行った．肺組織線維化の程度はAshcroftのscaleを用いて定量化し比較検討した[8]．Ashcroft scoreは組織標本において，肺の線維化の程度を顕微鏡下に0-8の段階に分け，各視野ごとにスコアリングをおこない，全視野をスコアリングしてスコアの平均により肺全体の線維化程度を評価する方法である．また投与開始後3，7，28日後に肺，肝における組織内ratHGF濃度をELISA法にて測定した．

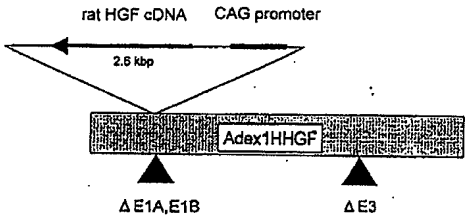

Fig. 1　Construction of Adex 1 CAHHGF.
△E1A，E1B，△E3はそれぞれvirus geneの領域の欠損部を示す．

結　果

adenovirus vector・BLM投与群における組織内HGF濃度は（Fig. 2）肺においては7日目に387.0±30.6ng／g tissueと上昇した．それに対しBLM・生食投与群では投与開始後28日目に400.9±44.2ng／g tissueとやや上昇したに過ぎなかった．肝臓におけるHGF濃度はadenovirus vector・BLM投与群において投与開始3日目に411.1±43.3ng／g tissueと上昇したが7日目には202.2±11.3ng／g tissueと低下した．BLM・生食投与群ではHGFの上昇は認めなかった．腎臓においてはadenovirus vector・BLM投与群，BLM・生食投与群ともに投与開始後3日目にHGF濃度の軽度の上昇が認められたが（それぞれ255±18.6，211±26.5ng／g tissue）28日目には両群でほぼ同じ値を示した．

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＊　びまん性肺疾患分科会　分科会員

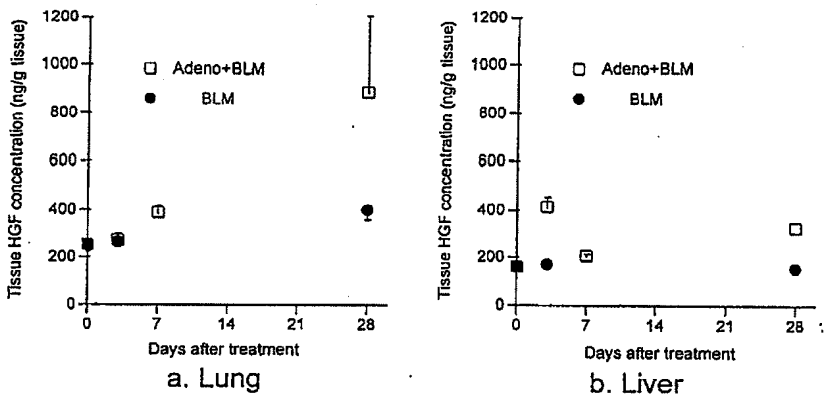

Fig. 2 肺と腎臓における HGF 組織内濃度

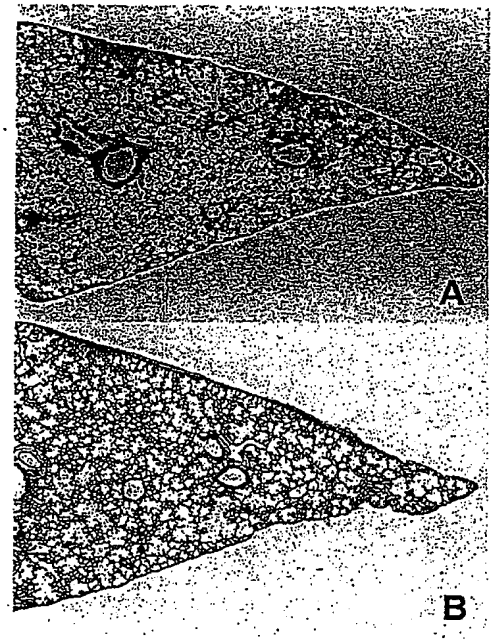

Fig. 3 Adex1CAHHGF・BLM 投与群と BLM・生食投与群の肺組織写真.
上が BLM・生食投与群（A），下が Adex1CAHHGF・BLM 投与群（B）を示す．BLM・生食投与群では胸膜下に線維化を認めるが，Adex1CAHHGF・BLM 投与群では線維化は著名に抑制されている．

投与開始後 28 日目の左肺の組織像を Fig. 3 に示す．BLM・生食投与群では主に胸膜下に楔状の線維化が認められ肺胞構造の破壊や滲出性の細胞浸潤も広汎に見られた．血管周囲・気道周囲では，胸膜下と同様に線維化が生じているところも認められた．adenovirus vector・BLM 投与群では炎症性変化はほとんど認められず，胸膜直下に一部線維化が見られるのみであり BLM による肺傷害は明らかに抑制されていた．

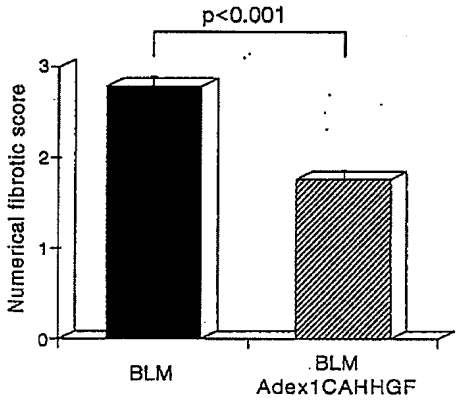

Fig. 4 Ashcroft score.
肺線維化の程度を示すアシクロフトスコアーは BLM・生食投与群に比し Adex1CAHHGF・BLM 投与群が有意に低値を示し線維化が抑制されていることを示している．

肺線維化の程度を Ashcroft の score によって定量化した（Fig. 4），投与開始後 4 週目で，adenovirus vector・BLM 投与群が 1.8 ±0.1 と BLM・生食投与群 2.8±0.1 より有意に低値を示し（$p<0.001$, ANOVA），線維化が抑制されていることを示している．

考案・結論

分子生物学の進歩により生体内において微量にしか存在しない生理活性物質を，疾患の治療に利用できる道が開かれた．我々はリコンビナント HGF 投与によって BLM 誘起性肺傷害を抑制しうる事を示している．しかしながら，リコンビナント蛋白は製造及び精製に莫大な費用がかかるため大量かつ持続的投与は経済的な問題点があり難しい．この点を克服するため我々は遺伝子導入によって生体内で HGF を大量かつ持続的に発現させる事を目的として ratHGF 遺伝子を組み込んだ Adenovirus vector を用いた遺伝子治療を行った．また遺伝子治療がリコンビナント蛋白投与より優れている点として，導入遺伝子によって発現した蛋白はその「生体」内で生成され修飾を受けるため，リコンビナント蛋白に比べより「自然」の蛋白に近い事があげられる．また持続投与となるため高い臓器内濃度が得られ，治療効果の増大が得られる可能性がある[9]．

今回我々の成績では，adenovirus vector：Adex 1 CA HHGF の腹腔内投与によって，投与後数日で肝臓における HGF 濃度が上昇しその後肺における HGF 臓器内濃度の上昇が見られた．HGF 濃度測定は抗 ratHGF 抗体を利用した ELISA 法によって行ったが，この抗体はマウスの内因性 HGF に対しても cross link することが知られており，肺における HGF の上昇は BLM による肺傷害に対するマウスの内因性 HGF の増大による可能性も否定できない．しかしながら，投与開始後 28 日目において adenovirus vector 投与群において，BLM 単独投与群より肺内 HGF 濃度が高値を示したことから導入遺伝子の発現によるものと解釈されよう．*In vivo* における導入遺伝子発現の解析が今後の課題である．

今回の我々の成績では adenovirus vector によって導入された ratHGF 遺伝子によって発現した HGF が，リコンビナント HGF を投与したときと同様かそれ以上に BLM 誘起性肺傷害を抑制し得ることが示され，遺伝子導入による肺線維性の新しい治療法の可能性を示している．

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Gene Therapy by Adenovirus vector having HGFcDNA on Medicament-induced Lung Injury

For the purpose of continuous expression of HGF in mouse models with injured lung at high concentration and for a long during, we have here tried the gene transfection using Adenovirus vector having rat HGFcDNA integrated thereto.

According to the results of our investigations here, several days after Adenovirus vector, Adex 1 CAHHGF, was intraperitoneally administered to mouse, the HGF level in liver of the mouse was increased, and then the increase of the HGF level in the lung was observed. The HGF level was determined according to ELISA using a anti-ratHGF antibody. Because the antibody cross-linkingly reacts with an endogenous rat HGF, the possibility was not denied that the increase of the HGF level in the lung might be due to the increase of the endogenous rat HGF against the lung injury induced by Bleomycin (BLM). However, on 28th day after the administration of Adenovirus vector, the Adenovirus vector-administered group indicated significantly higher HGF level in the lung than the only BLM-administered group, which shows that the increase of the HGF level in the lung was due to the expression of the gene transfected according to Adenovirus vector. Thus, the investigation of the gene expression in vivo will be the next problem.

The results of our investigations here have

revealed that HGF expressed by the rat HGF gene transfected according to Adenovirus vector inhibited the lung injury induced by BLM in the same level as or more than the recombinant HGF administered. Thus, these results show the possibility of a new therapy for pulmonary fibrosis according to a gene transfection.

Gene Transfection of Hepatocyte Growth Factor Attenuates Reperfusion Injury in the Heart

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Background. Hepatocyte growth factor (HGF), a ligand for the c-Met receptor tyrosine kinase, plays a role as organotrophic factor for regeneration of various organs. HGF has an angiogenic activity and exhibits a potent antiapoptotic activity in several types of cells. Although HGF and the c-Met/HGF receptor are expressed in the heart, the role of HGF in the heart has remained unknown.

Methods. After we analyzed changes in expression of endogenous HGF and c-Met mRNA levels in the rat left ventricle after myocardial infarction, the human HGF gene in hemagglutinating virus of Japan (HVJ)-liposome was transfected into the normal whole rat heart. Three days after transfection, the heart was subjected to global warm ischemia and subsequent reperfusion, followed by assessment of its cardiac functions.

Results. Both HGF and c-Met/HGF receptor mRNAs were expressed in adult rat heart, and c-Met/HGF receptor mRNA was upregulated in response to myocardial infarction. HGF-transfected heart showed significant increase of human HGF protein level in the heart. Cardiac functions in terms of the left ventricular developed pressure, maximum dp/dt, and pressure rate product in hearts with HGF gene transfection were significantly superior to those in control hearts. In addition, leakage of creatine phosphokinase in the coronary artery effluent in hearts with HGF gene transfection was significantly lower than that in control hearts.

Conclusions. These data indicated that both HGF and c-Met/HGF receptor mRNAs were upregulated in response to myocardial ischemic injury, and that HGF is likely to have a cytoprotective effect on cardiac tissue, presumably through the c-Met/HGF receptor.

(Ann Thorac Surg 1999;67:1726–31)

Recent advances in myocardial protection have improved the clinical results of open heart surgery. However, severely critical cases associated with compromised heart, such as failing heart or postischemic conditions, still occur, and thus, further attempts to improve myocardial protection should be addressed. Recent studies have revealed the importance of an endogenous myocardial protective system against ischemia-reperfusion injury [1, 2]. For an advanced strategy for myocardial protection, therefore, implementation of such a system appears to be essential.

Hepatocyte growth factor (HGF), originally identified and cloned as a potent mitogen for hepatocytes [3], exhibits mitogenic, motogenic, and morphogenic activities for a wide variety of cells [4, 5]. In addition, HGF is an angiogenic factor and exerts a potent anti-cell death effect on several types of cells [6]. These biological activities of HGF were found to be initiated by autophospholylation of proto-oncogene c-Met, the receptor tyrosine kinase for HGF [7]. Although c-Met is induced in the embryonic heart [8] and constitutionally expressed in the adult heart, especially in coronary endothelial cells [9], the role of HGF as an endogenous protective factor in the heart has not yet been determined.

In this study, at first we analyzed the expression of endogenous HGF and c-Met in the heart after myocardial infarction, and next determined whether HGF attenuates ischemia-reperfusion injury in the heart by using in vivo gene transfection of human HGF into the whole heart with the hemagglutinating virus of Japan (HVJ)-liposome method [10].

Material and Methods

Acute Myocardial Infarction Model

Twelve Wistar male rats were used for this study. Humane animal care complied with the "Principles of Laboratory Animal Care" formulated by the National Society for Medical Research and the "Guide for the Care and Use of Laboratory Animals" prepared by the Institute of Laboratory Animal Resource and published by the National Institutes of Health (NIH Publication No. 86-23, revised 1985). Acute myocardial infarction was induced as described elsewhere [11]. Briefly, the rats (8 weeks after birth) were anesthetized with sodium pentobarbital, and positive-pressure respiration was applied through

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an endotracheal tube. The thorax was opened at the fourth left intercostal space, and the left coronary artery was completely ligated 3 mm distal to its origin by means of a 7-0 polypropylene ligature.

Expression of HGF and c-Met Receptor mRNA

Expression of HGF and c-Met mRNAs was analyzed by reverse transcription-polymerase chain reaction (RT-PCR). The following primers were used: 1) for both rat and human HGF (Gene bank accession number D90102): forward primer, 5′-TTG GCC ATG AAT TTG ACC TC-3′ and reverse primer, 5′-ACA TCA GTC TCA TTC ACA GC-3′; 2) for human HGF (Gene bank accession number X16323): forward primer, 5′-GCC TCT GGT TCC CCT TCA ATA G-3′ and reverse primer, 5′-CCA TGA GAC CTC GAT AAC TCT CC-3′; 3) for c-Met: forward primer (Gene bank accession number J02958), 5′-TGT GCA TTC ACT AAA TAT GT-3′ and reverse primer, 5′-GTC CCA GCC ACA TAT GGT CA-3′. PCR conditions were as follows: denaturation at 94°C for 30 seconds, followed by annealing at 55°C for 60 seconds and extension at 72°C for 60 seconds. PCR products were subjected to electrophoresis in 1% agarose gel and visualized by ethidium bromide staining.

Transfection of Human HGF Gene Into the Heart

cDNA of human HGF was inserted into the Not I site of the pUC-SRα expression vector [12]. The preparation of the liposome complex with hemagglutinating virus of Japan (HVJ) is described elsewhere [13].

The donor rats were anesthetized and the hearts arrested by infusion of cardioplegic solution into the abdominal aorta for coronary perfusion. After the hearts were removed, approximately 0.7 mL of HVJ-liposome-plasmid complex (including 50 μg of cDNA of human HGF) was infused into the aorta of the resected hearts for coronary perfusion. The expression vector containing HGF cDNA or the empty vector was transfected into the hearts, and 6 animals were used in each experimental group. The hearts were then incubated on ice for 10 minutes and transplanted into the abdomen of recipient rats of the same strain. The transplantation was performed by anastomosing the descending aorta to the abdominal aorta and the pulmonary artery to the inferior vena cava in an end-to-side fashion [14]. Total ischemia time was 45 ± 5 minutes. The transplanted hearts were resuscitated to spontaneous and continuous beating after restoring of blood flow.

Enzyme-Linked Immunosorbent Assay for Human HGF

Human HGF in cardiac tissue was measured by means of enzyme-linked immunosorbent assay (ELISA) using anti-human HGF monoclonal antibody (Institute of Immunology, Tokyo, Japan). The human HGF ELISA system also specifically detects human HGF but not rat HGF [15].

Histopathology

After global ischemia followed by reperfusion with the Langendorff perfusion system, the tissue specimens were obtained as transverse sections from the perfused heart 5 mm basal from the apex. The tissue specimens were frozen in an OCT compound. Frozen sections were stained with hematoxylin and eosin.

Measurement of Cardiac Function of the Transplanted Rat Heart

The transplanted heart was excised and mounted on the aortic cannula on the perfusion apparatus 3 days after gene transfection. The coronary arteries were perfused according to the Langendorff technique at perfusion pressures of 110 cm H_2O as described elsewhere [16]. The heart was housed in a controlled heart chamber maintained at 37°C. During a 10-minute washout period after cannulation, an intraventricular balloon was inserted into the left ventricle through the mitral valve. The balloon was filled with fluid and attached to a pressure transducer, while the volume of the balloon was adjusted by means of a watertight microsyringe. Thirty minutes after the aortic cannulation, heart rate (HR), left ventricular developed pressure (LVDP), max dp/dt, and coronary flow (CF) were measured at constant left ventricular end-diastolic pressure. The left ventricular end-diastolic pressure was initially set at 10 cm H_2O. The hearts were then subjected to global ischemia at 37°C for 30 minutes followed by 30 minutes of reperfusion. The balloon was deflated during ischemia and the indices of cardiac function were measured 30 minutes after reperfusion. The coronary effluent was collected in chilled vials to measure creatine phosphokinase (CPK) during a 5-minute period after reperfusion.

Statistical Analysis

All values are expressed as the mean ± standard deviation. Statistical differences in the data for functional recoveries and enzyme activity were evaluated by unpaired Student's t test for comparisons between two means. A p value of less than 0.05 was considered statistically significant.

Results

Changes in HGF and c-Met Receptor mRNA Expression After Myocardial Infarction

Thirty-six percent of the infarcted rats died within 6 hours after the operation. No technical failure occurred during this study. We first analyzed changes in the expression of HGF and c-Met/HGF receptor mRNAs in the left ventricle of the infarcted heart by using RT-PCR after left coronary artery ligation. Three rats were used at each time point: 24 hours, 3 days, and 7 days after ligation. RT-PCR was performed using specific sets of primers that detect rat HGF and c-Met receptor mRNAs. The RT-PCR product derived from rat HGF mRNA was seen in the normal rat left ventricle, indicating that HGF mRNA is expressed in it (Fig 1). The expression of HGF mRNA detected by RT-PCR analysis did not change remarkably, and the inconsequential change in the ex-

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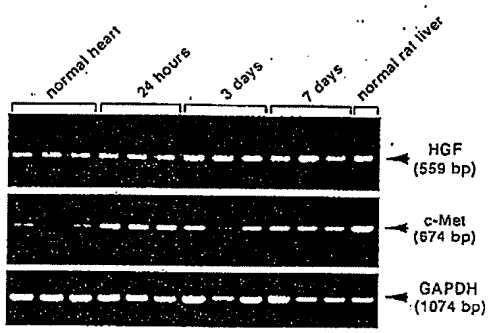

Fig 1. Changes in HGF and c-Met/HGF receptor mRNA levels in rat hearts after myocardial infarction. Total RNA was prepared from the left ventricle at 1, 3, and 7 days after left coronary artery ligation, and three rats from each group were used. RT-PCR products were subjected to electrophoresis and visualized by ethidium bromide staining. Expression of glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) mRNA was used as an internal control.

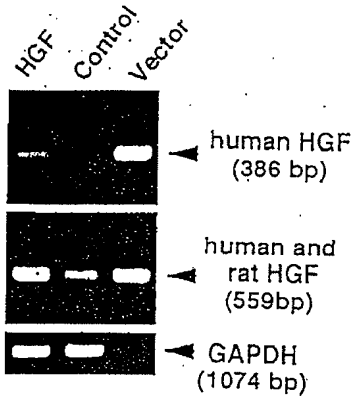

Fig 2. Expression of human and rat HGF mRNAs in rat hearts transfected with human HGF gene. Expression of HGF mRNA was analyzed by means of RT-PCR, using total RNA prepared from whole heart 3 days after in vivo gene transfection of human HGF (left lane) *or empty vector* (middle lane). *A naked expression vector containing human HGF cDNA was used as a positive control for PCR* (right lane).

pression of HGF mRNA was seen 3 and 7 days after ligation.

On the other hand, c-Met receptor mRNA expression in the left ventricle increased and reached its highest level 24 hours after ligation. The c-Met/HGF receptor mRNA level was seen to decrease thereafter, but 3 and 7 days after ligation, it was still higher than that seen in the normal rat left ventricle (Fig 1). Although the c-Met receptor mRNA level 3 days after ligation in one animal was lower than that seen 24 hours after ligation, we could not come to a definitive conclusion that the decrease was physiologically significant. Nonetheless, these expression patterns suggest that both HGF and c-Met/HGF receptor mRNAs are expressed in adult rat heart, and c-Met mRNA is upregulated in response to myocardial ischemic injury caused by myocardial infarction.

In Vivo HGF Gene Transfection Into the Heart

To analyze the expression of the transgene of human HGF, total RNA was prepared from the transplanted whole hearts 3 days after HGF gene transfection, and human HGF mRNA expression was then analyzed by means of RT-PCR using a primer set that specifically detects human HGF mRNA but not rat HGF mRNA. The RT-PCR product derived from human HGF mRNA was specifically detected in the rat heart transfected with the expression vector for human HGF, indicating that transfected human HGF cDNA was specifically expressed in the heart. On the other hand, RT-PCR analysis using a primer set that detects both rat and human HGF mRNAs indicated that the total HGF mRNA level in the heart treated with the HGF gene transfection was higher than that seen in the heart treated with the empty vector (Fig 2). Moreover, human HGF protein content in the transfected hearts was measured by means of ELISA using anti-human HGF monoclonal antibody. The content of human HGF in the cardiac tissues obtained from the heart treated with the HGF gene transfection was 0.56 ± 0.11 ng/g tissue. In contrast, human HGF was undetect-

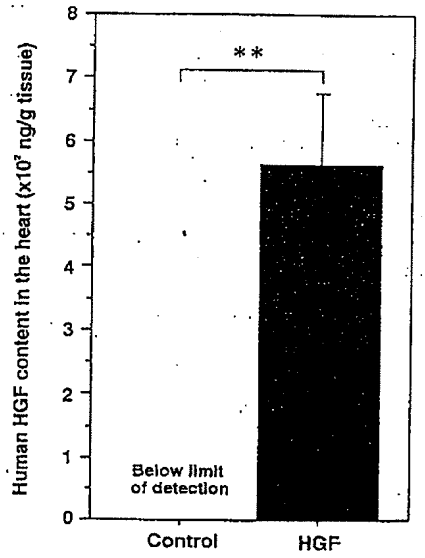

Fig 3. Expression of human HGF protein measured by means of ELISA in rat hearts transfected with human HGF. $^{**}p < 0.001$.

Table 1. Parameters of Cardiac Function Before Global Ischemia in Langendorff Perfusion

Group	n	HR (beats/min)	LVDP (mm Hg)	max dp/dt (mm Hg/sec)	CF (mL/min)
HGF	6	230 ± 45	80 ± 6.8	1023 ± 86	12.8 ± 2.4
Control	6	226 ± 56	76 ± 8.4	996 ± 92	13.4 ± 3.5

Data are expressed as the mean ± the standard deviation. There was no significant difference in any parameters between both groups before global ischemia.

CF = coronary flow; HGF = Hepatocyte growth factor gene-transfected group; HR = heart rate; LVDP = left ventricular developed pressure; n = number of samples.

able in the cardiac tissues obtained from the hearts treated with transfection of an empty vector (Fig 3).

Recovery of Cardiac Function After Global Ischemia

On the basis of these results, global warm ischemia followed by reperfusion was performed on the isolated hearts 3 days after the gene transfection. Cardiac function was analyzed before and after global warm ischemia and reperfusion. In this experiment, no significant differences in HR, LVDP, maximum dp/dt, or CF were seen before global ischemia in either experimental group (Table 1).

Evaluation of recovery of cardiac function of the hearts transfected with the empty vector showed that LVDP had decreased to 63 ± 3%, maximum dp/dt to 63 ± 6%, and pressure rate product (PRP) to 61 ± 5% of the values observed before global warm ischemia (Fig 4), thus indicating the onset of cardiac dysfunction. In contrast, the corresponding values of the hearts transfected with the human HGF gene decreased to only 86 ± 3%, 83 ± 5%, and 85 ± 4% of that before global warm ischemia. HR and CF of the hearts transfected with the empty vector, respectively, decreased to 78% and 81% of the values obtained before global ischemia. The corresponding values of the hearts transfected with the human HGF gene were 86% and 94%, respectively. These values showed no significant differences between the hearts with transfection of the human HGF gene and of the empty vector.

CPK activity was not detected in the coronary effluent before global ischemia in either experimental group (data not shown), while after ischemia and reperfusion injury, CPK activity in the coronary effluent obtained from the empty vector transfected heart increased to 72 ± 24 × 10^{-3} IU/5 min. In contrast, CPK activity was undetectable in the coronary effluent obtained from the HGF gene-transfected hearts (Fig 5).

Histological findings of the HGF gene-transfected heart showed slight eosinophilic staining in cardiac myocytes and mild interstitial edema. On the other hand, the findings of the empty vector transfected heart showed strong eosinophilic staining in cardiac myocytes, hypercontracted cardiac myocytes, and severe interstitial edema (Fig 6).

Comment

Myocardial ischemia and subsequent reperfusion induce myocardial cellular injury, including apoptosis and/or necrosis in cardiac myocytes and coronary endothelial cells [17]. Cell death in cardiac myocytes is in turn accompanied by myocardial dysfunction. Prevention of cellular injury and death attenuates the magnitude of myocardial dysfunction. Therefore, application of growth factors that target cardiac myocytes and/or endothelial cells may be potentially beneficial for the prevention of myocardial dysfunction.

Previous studies have shown that the expression of both HGF and c-Met are upregulated in the adult rat heart after ischemia-reperfused heart, at least in the coronary endothelial cells. The results presented in this first study were mostly compatible with data of these previous reports [9]. Therefore, strong density of RT-PCR products band derived from c-Met mRNA 24 hours after left coronary ligation using ethidium bromide staining indicates at least the induction of it. In a more recent study of ours, we found that both highly purified cardiac endothelial cells and mature cardiac myocytes express the c-Met/HGF receptor and HGF mRNA. These results

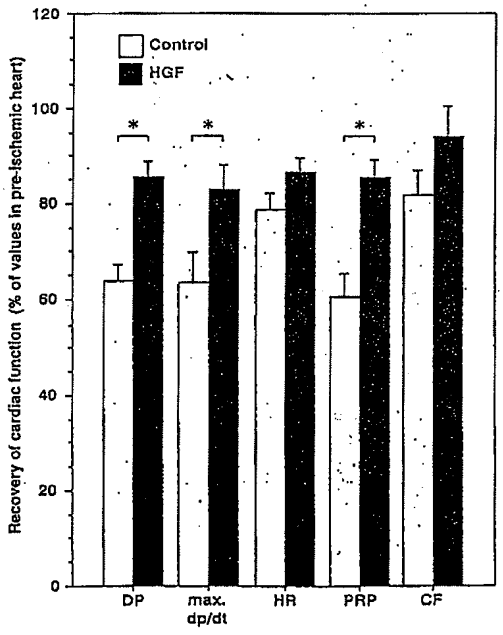

*Fig 4. Enhancement of recovery of cardiac function from ischemia-reperfusion injury as a result of cardiac transfection of the HGF gene. The heart was removed 3 days after treatment for gene transfection and subjected to global warm ischemia for 30 min and subsequent reperfusion. *p < 0.05.*

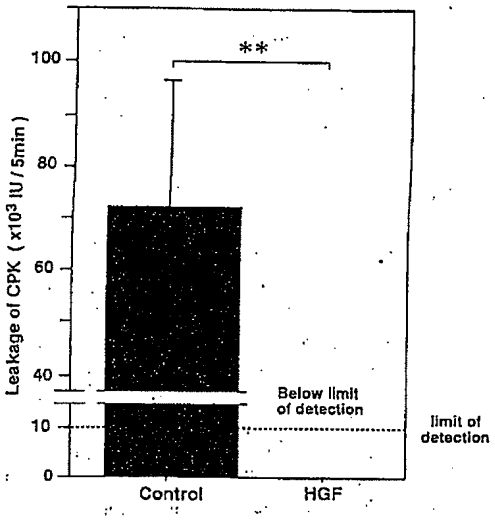

*Fig 5. Suppression of leakage of CPK from the cardiac myocytes in the rat heart transfected with the HGF gene. CPK activity was measured in the coronary effluent obtained for 5 min after reperfusion. **p < 0.001.*

imply that HGF plays certain physiological roles in both cardiac myocytes and endothelial cells.

Although we showed in this second study that HGF transducted from cardiac tissue-directed gene transfection might enhance recovery from ischemia-reperfusion injury, the mechanisms remain to be addressed. Previous studies have demonstrated that HGF attenuates endothelial cell death [18]. The additional finding that cardiac endothelial cells express the c-Met/HGF receptor suggested that HGF might exert cardiotrophic action at least through targeting cardiac endothelial cells. On the other hand, it is also highly probable that HGF expresses its cardiotrophic function through its direct action on mature cardiac myocytes. We recently obtained evidence that HGF suppressed cell death of highly purified mature cardiac myocytes isolated from the rat hearts in vitro after cellular injury caused by hypoxia or hydrogen peroxide-induced oxidant stress (manuscript in preparation). Moreover, the recovery of left ventricular-developed pressure (LVDP) in rats given HGF gene transfection was higher than that in control rats in spite of no significant changes in coronary flow (CF), while the leakage of CPK from the heart of rats with HGF gene transfection was much lower than that seen in control rats. These results strongly suggest that HGF exerts a cardiotrophic effect through targeting both cardiac myocytes and coronary endothelial cells.

We previously showed that the intracoronary infusion of a gene using the HVJ-liposome method resulted in the efficient transduction of a gene into the entire rat heart. It was found that the gene for β-galactosidase was overexpressed in more than 50% of myocytes in the whole rat heart [10]. The detection of human HGF mRNA by means of RT-PCR and human HGF protein by means of ELISA using the anti-human HGF monoclonal antibody to human HGF but not to rat HGF also confirmed the successful transfection of the HGF gene. It can therefore be assumed that HGF is effectively expressed in cardiac tissue by means of this HVJ-liposome method and that it attenuates myocardial dysfunction through the c-Met/HGF receptor.

According to previous reports, pretreatment with recombinant HGF through intraperitoneal or intravenous injection from 6 to 24 hours before acute insult achieved its optimal function in other organs such as liver, kidney, and lung [6, 19]. This finding indicates that pretreatment just before global ischemia by means of exogenous injection of recombinant HGF might have little effect on ischemia-reperfusion injury in the myocardium. In the study presented here, the physiological experiment on isolated transfected hearts was performed 3 days after gene transfection, although transgene product should be

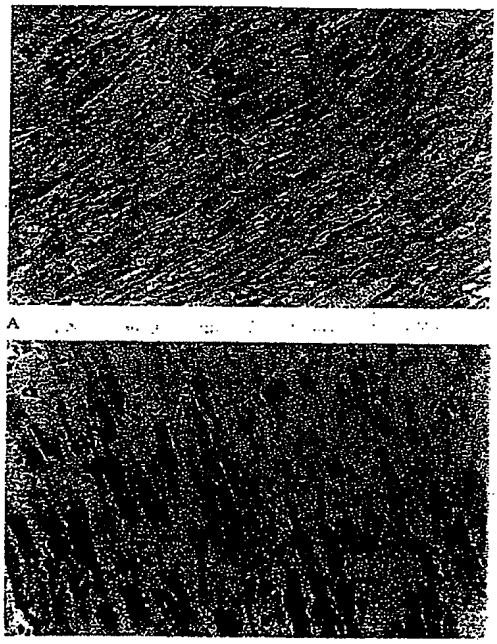

Fig 6. Representative histological findings of the in vivo gene-transfected heart after global warm ischemia followed by reperfusion with the Langendorff perfusion system using crystalloid solution. The damage to the myocardial tissue in the HGF gene-transfected heart (A) was less than to it in the empty vector-transfected heart (B) after global warm ischemia followed by reperfusion (×200).

translated within 12 to 48 hours. Therefore, the transfected hearts received chronic exposure to HGF for at least 24 hours. The pronounced effect on cytoprotection of the transfected heart against ischemia-reperfusion injury may reflect the cumulative effect of continuous biosynthesis and chronic exposure to HGF.

It should be emphasized that HGF has been shown to have a potent anti-cell death effect both in vitro and in vivo for various types of cells, including hepatocytes [6], renal tubular cells [20], vascular endothelial cells [18], and neurons [21]. Therefore, HGF probably exerted its cardiotrophic action through its anti-cell death function in the experimented model used in the present study.

In conclusion, we obtained evidence that the c-Met/HGF receptor gene was expressed in both the normal and the infarcted heart, and that HGF introduced by means of cardiac transfection of the HGF gene attenuated myocardial dysfunction caused by ischemia-reperfusion injury. This study is the first to demonstrate the cardiotrophic activity of HGF in vivo, and our ongoing study is directed at clarification of the molecular and cellular mechanisms for the action of HGF functioning as a cardiotrophic growth factor.

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Therapeutic Angiogenesis Induced by Human Hepatocyte Growth Factor Gene in Rat and Rabbit Hind Limb Ischemia Models: Pre-clinical Study to Treat Peripheral Arterial Disease

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Running title: Therapeutic angiogenesis induced by HGF gene transfer

Keywords: peripheral vascular disease, endothelium, angiogenesis, gene therapy, HGF

In this study, we examined the feasibility of gene therapy using HGF to treat peripheral arterial disease. Intramuscular injection of human HGF plasmid resulted in a significant increase in capillary density and blood flow, accompanied by the detection of human HGF. Importantly, its degree by HGF plasmid was significantly greater than recombinant HGF. Even in rabbit hindlimb ischemia model, intramuscular injection of HGF plasmid produced significant augmentation of collateral vessel development, blood flow and blood pressure ratio of ischemic to normal limb. Overall, intramuscular injection of naked HGF plasmid induced therapeutic angiogenesis in rat and rabbit ischemic hind limb model.

Abstract

Hepatocyte growth factor (HGF) exclusively stimulates the growth of endothelial cells without replication of vascular smooth muscle cells, and acts as a survival factor against endothelial cell death. Recently, a novel therapeutic strategy for ischemic diseases using angiogenic growth factors to expedite and/or augment collateral artery development has been proposed. We have previously reported that intra-arterial administration of recombinant HGF induced angiogenesis in rabbit hindlimb ischemia model. In this study, we examined the feasibility of gene therapy using HGF to treat peripheral arterial disease rather than recombinant therapy due to its disadvantages. Initially, we examined the transfection of "naked" human HGF plasmid into rat hindlimb ischemia model. Intramuscular injection of human HGF plasmid resulted in a significant increase in blood flow as assessed by laser doppler imager, accompanied by the detection of human HGF protein. A significant increase in the number of capillary density was also increased in rats transfected with human HGF as compared to control vector in a dose-dependent manner ($P<0.01$). Importantly, at 5 weeks after transfection, the degree of angiogenic property by transfection of HGF plasmid was significantly greater than that caused by a single injection of recombinant HGF. Toward to human gene therapy, we also employed rabbit hindlimb ischemia model as preclinical study. Naked HGF plasmid was intramuscularly injected in the ischemic hindlimb of rabbits, to evaluate its angiogenic activity. Intramuscular injection of HGF plasmid once on day 10 after surgery produced significant augmentation of collateral vessel development on day 30 in the ischemic model, as assessed by angiography ($P<0.01$). Serial angiograms revealed progressive linear extension of collateral arteries from the origin stem artery to the distal point of the reconstituted parent vessel in HGF-transfected animals. In addition, a significant increase in blood flow as assessed by doppler flow wire and in the blood pressure ratio of ischemic limb to normal limb was observed in the rats transfected with HGF plasmid as compared to rabbits transfected with control vector ($P<0.01$).

Overall, intramuscular injection of naked human HGF plasmid induced therapeutic angiogenesis in the rat and rabbit ischemic hind limb model, as potential therapy for peripheral arterial disease.

Introduction

Critical limb ischemia is estimated to develop in 500 to 1000 individuals per million per year (1). In a large proportion of these patients, the anatomical extent and the distribution of arterial occlusive disease make the patients unsuitable for operative or percutaneous revascularization. Thus, the disease frequently follows an inexorable downhill course (2,3). Of importance, there is no optimal medical therapy for critical limb ischemia, as the Consensus Document of the European Working Group on Critical Limb Ischemia concluded (1). Therefore, novel therapeutics are required to treat these patients. In the pathophysiology of the disease, in the presence of obstruction of a major artery, blood flow to the ischemic tissue is often dependent on collateral vessels. When spontaneous development of collateral vessels is insufficient to allow normal perfusion of the tissue at risk, residual ischemia occurs. Recently, the efficacy of therapeutic angiogenesis using VEGF (vascular endothelial growth factor) gene transfer has been reported in human patients with critical limb ischemia and myocardial ischemia (4-6). Thus, the strategy for therapeutic angiogenesis using angiogenic growth factors should be considered for the treatment of patients with critical limb ischemia or myocardial infarction. Most of the studies have used VEGF, also known as vascular permeability factor, as well as a secreted endothelial-cell mitogen. The endothelial cell specificity of VEGF has been considered to be an important advantage for therapeutic angiogenesis, as endothelial cells represent the critical cellular element responsible for new vessel formation (7,8).

From this viewpoint, hepatocyte growth factor (HGF) is also of particular interests, as HGF exclusively stimulated the growth of endothelial cells without replication of vascular smooth muscle cells (VSMC) (9-11). Indeed, others and we previously reported that HGF is a potent angiogenic growth factor and useful for the aim of therapeutic angiogenesis in rabbit ischemia model (12,13). Unexpectedly, the mitogenic activity of HGF is more potent than that of VEGF in human aortic endothelial cells in vitro as well as rabbit hindlimb ischemia model in vivo (10,13). Moreover, its specific receptor, c-met, have been shown to be up regulated in the ischemic tissue (14). Therefore, we reasoned that HGF should be a potential therapeutic angiogenic growth factor, in addition to VEGF. In this study, we examined the feasibility of gene therapy using HGF to treat

peripheral arterial disease rather than recombinant therapy as the following reasons: 1) It is the potential requirement to maintain an optimally high and local concentration over time. This issue may be most critical in the case of arterial gene therapy. In the case of therapeutic angiogenesis, it may be preferable to deliver a lower dose over a period of several days or more from an actively expressing transgene in the iliac artery, rather than a single or multiple bolus doses of recombinant protein to avoid the side-effects; 2) There is the matter of economics; namely, which therapy would ultimately cost more to develop, implement, and reimburse, particularly for those indications requiring multiple or even protracted treatment ? 3) The feasibility of a clinical trial of recombinant protein is currently limited by the lack of approved or available quantities of human quality grade of recombinant protein, due in large part to the nearly prohibitive cost of scaling up from research grade to human quality recombinant protein. Here, we demonstrated pre-clinical studies that injection of "naked" HGF plasmid induced therapeutic angiogenesis to treat peripheral arterial disease in rat and rabbit hindlimb ischemia model toward to human clinical trial.

Methods

Experiment 1: rat hindlimb ischemic model

Construction of plasmids

To produce a HGF expression vector, human HGF cDNA (2.2 kb) was inserted into a simple eucaryotic expression plasmid that utilizes the cytomegalovirus (CMV) promoter/enhancer. This promoter/enhancer has been used to express reporter genes in a variety of cell types and can be considered to be constitutive. Downstream from the HGF from the HGF cDNA is the SV40 polyadenylation sequence. The control vector used as a control was CMV expression vector plasmid, which did not contain HGF cDNA. We obtained luciferase gene expression vector driven by SV 40 promoter from a commercially available source (Promega Corporation, Madison, WI).

In vivo gene transfer using direct intramuscular injection approach

Sprague-Dawley rats (400-500 g; Charles River Breeding Laboratories) were anesthetized with an intraperitoneal injection of sodium pentobarbital (0.1 ml/100 mg). A longitudinal incision was then made, extending inferiorly from the inguinal ligament to a point just proximal to the patella. Through this incision, using surgical loupes, the operator dissected free the right femoral artery along its entire length; all branches of the femoral artery, including the inferior epigastric, deep femoral, lateral circumflex, and superficial epigastric arteries, were also dissected free (15). After dissection of the popliteal and saphenous arteries distally, the external iliac artery and all of the mentioned arteries were ligated with 4-0 silk (Ethicon). Finally, the right femoral artery was completely excised to create the ischemic limb model, from its proximal origin as a branch of the external iliac artery to the point distally where it bifurcates to form the saphenous and popliteal arteries. Excision of the femoral artery results in retrograde propagation of thrombus and occlusion of the external iliac artery. Excision of the femoral artery 1 cm below the peritoneum created an ischemia model. Consequently, blood flow to the ischemic limb was dependent on collateral vessels developing from the internal iliac artery.

Design 1: Injection of "naked" human HGF (100, 250 or 500 ug/body) or control vector (500 ug/body) was also carefully injected directly into the right ischemic limb of rats with a 27 G needle (Terumo) immediately after surgery. Injection volume of plasmid was 100 μl.

Design 2: In design 2, we administered a single injection of 25 or 50 μg recombinant human HGF (rhHGF) locally (intra-arterially into the right ischemic limb) on day 0 after the operation. The experimental procedure was the same as described above. Animals received the first intra-arterial bolus of rhHGF (25 or 50 μg/body) or vehicle (0.1 % rabbit serum albumin) administered as a bolus over 1 minute through a cannula positioned in the internal iliac artery of the ischemic limb.

Analysis of Luciferase activity

Firefly luciferase activity was measured using a luciferase assay system (PicaGene™; Toyo-Inki, Tokyo, Japan). Rats were sacrificed at 5 days after transfection of luciferase gene with direct transfection of "naked" plasmid by direct injection into hindlimb. The tissue samples (200

mg around injection site) were rapidly frozen in liquid nitrogen, and homogenized in a lysis buffer. The tissue lysates were briefly centrifuged (3000 rpm, 10 min), and 20 μl of supernatant was mixed with 100 μl of luciferase assay reagents. The measurements of the luminescent reaction were started at 5 sec after addition of sample. The counting lasted for 10 sec, and the counts in 10 sec were used for calculation of luciferase activity (16).

Measurement of HGF concentration in hindlimb

To document successful transfection of HGF vector into the hindlimb, we examined the production of human immunoreactive HGF (11,17). Four days after transfection, the hindlimb of rats transfected with HGF or control vector were promptly removed without excess fat after perfusion via the apex with saline, frozen in liquid nitrogen, and then stored at -70 °C until use. On the day of extraction, the tissue was thawed at 4 °C, weighed, and homogenized by polytron in assay solution. Each specimen was centrifuged at 20,000 X g for 30 minutes at 4 °C, to remove the lysates. The concentration of HGF in the hindlimb was determined by enzyme-immunoassay using anti-human HGF antibody, as described previously (11,17). Briefly, rabbit anti-rat or anti-human HGF IgG was coated on 96-well plates (Corning, NY) at 4°C for 15 hours. After blocking with 3% bovine serum albumin in phosphate-buffered saline (PBS), conditioned medium was added to each well, and the preparation was incubated for 2 hours at 25 °C. Wells were washed three times with PBS containing 0.025% Tween 20 (PBS-Tween), biotinylated rabbit anti-human HGF IgG was added and the preparation was incubated for 2 hours at 25 °C. After washing with PBS-Tween, wells were incubated with horseradish peroxidase-conjugated streptavidin-biotin complex in PBS-Tween. The enzyme reaction was initiated by adding substrate solution composed of 2.5 mg/ml *o*-phenylenediamine, 100 mM sodium phosphate, 50 mM citric acid, and 0.015% H_2O_2. The enzyme reaction was halted by adding 1 M H_2SO_4, and absorbance at 490 nm was measured. The antibody against human HGF reacts with only human HGF, and not with rat HGF (21). Rat immunoreactive HGF in the rat hindlimb was also measured by EIA using anti-rat HGF antibody, as the antibody against rat HGF reacts with only rat HGF, and not with human HGF (17).

Measurement of blood flow by laser doppler image

The measurement of blood flow with Laser Doppler Imager (LDI) has been previously described (18,19). As it was clearly demonstrated that Laser Doppler flow velocity well correlates with the capillary density (18,19), we measured the cardiac blood flow by means of a Laser Doppler blood flowmeter (Laser Doppler Imager, Moor Instruments, England). Indeed, we confirmed that the blood flow measured by LDI was well correlated with the capillary density (data not shown). Excess hairs were removed by depilatory cream from the limb before imaging, and rats were placed on a heating plate at 37 °C to minimize temperature variation. Consecutive measurements were obtained over the same region of interests (leg and foot). LDI uses a 12-mW helium-neon laser beam that sequentially scans a 5X5 cm surface area with extremely high speed to be able to measure the blood flow in the ischemic hindlimb. The blood flow at 1 mm under the surface can be measured. While scanning, blood cells that are in motion shift as the frequency of projection light according to the Doppler principle. Upon the termination of scanning, a color-coded image representing blood flow distribution is displayed on a monitor. The perfusion signal is subdivided into 14 different intervals, and each interval is displayed in a separate color. Low or no perfusion is displayed as dark blue, whereas the highest perfusion intervals is displayed as white. The stored perfusion values behind the color-coded pixels remain available for the data analysis. Before measuring, rats were anesthetized, intubated and connected to a respirator. Perfusion analyses were performed sequentially; a) the ischemic hindlimb transfected with control vector, b) the ischemic hindlimb transfected with HGF vector. These laser images were quantitatively converted into histograms that represented the amount of blood flow on the x-axis and the number of pixels on the y-axis in the traced area. The average blood flow in each histogram was calculated to be evaluated.

Measurement of capillary density

Alkaliphosphatase staining was used as specific markers for endothelial cells in paraffin-

embedded sections, as previously described (20,21). To analyze the number of vessels in the ischemic hindlimb transfected with HGF vector or control vector, rats were sacrificed and the muscles were removed. Three individual sections from the middle of the transfected muscle were analyzed. The number of vessels was counted under a light microscopy (magnification, x 100) in a blinded manner. The total number of vascules in each section was summed and expressed as number per section. At least ten individual sections were evaluated on each muscle. The areas, in which the number of vascules was quantified, were randomly selected in the injected site and around the injected site. The animals were coded so that the analysis was performed without any knowledge of which treatment for each individual had received. The reproducibility of the results was assessed. Intraobserver variability was determined from triplicate measurements performed by one observer for all sections. The mean ± SD difference among measurements made by the same observer was 1.8 ± 0.2 %. Interobserver variability was determined from measurements of 10 randomly selected sections performed by the second observer in addition to the first one. The numerical difference between the measurements made by two observers was 2.1 ± 0.8 %. These observers were blinded to other data concerning the rats, as well as to the results of other observers.

Experiment 2: rabbit hindlimb ischemic model

Rabbit ischemic hind limb model

The physiological response to transfection of human HGF vector was also investigated in the rabbit ischemic hind limb model, described in previous reports (21). Briefly, male New Zealand White rabbits (3.5 to 4.0 kg) (Kitayama Rabes, Japan) were anesthetized with a mixture of xylazine (5 mg/kg) and ketamine (50 mg/kg). A longitudinal incision was then made, extending inferiorly from the inguinal ligament to a point just proximal to the patella. Through this incision, using surgical loupes, the operator dissected free the left femoral artery along its entire length; all branches of the femoral artery, including the inferior epigastric, deep femoral, lateral circumflex, and superficial epigastric arteries, were also dissected free. After dissection of the popliteal and saphenous arteries distally, the external iliac artery and all of the mentioned arteries were ligated

with 4-0 silk (Ethicon). Finally, the left femoral artery was completely excised to create the ischemic limb model, from its proximal origin as a branch of the external iliac artery to the point distally where it bifurcates to form the saphenous and popliteal arteries. Excision of the femoral artery results in retrograde propagation of thrombus and occlusion of the external iliac artery. Excision of the femoral artery 1 cm below the peritoneum created an ischemia model. Consequently, blood flow to the ischemic limb was dependent on collateral vessels developing from the internal iliac artery.

Four separate injections of 250 or 500 μg human HGF vector (total 1 or 2 mg) locally (intra-muscular into the ischemic limb) were performed. Ten days after surgery (day 10) and after measurement of baseline body weight as well as baseline noninvasive and invasive measurements of hemodynamic parameters, the animals received the intra-muscular injection of human HGF "naked" plasmid vector or control plasmid lacking HGF gene (2 mg total) through a 27 gauge needle injection (Terumo, Atsugi, Japan) positioned above and below knee of the ischemic limb.

Quantitative angiography

The angiographic luminal diameter of the internal iliac artery in the ischemic limb at baseline and after drug infusion was determined on day 0, 21 and 35 by previously described techniques (12,13). Briefly, morphometric analysis of collateral vessel development in the ischemic limb was performed in 4-second angiograms recorded after injection of contrast medium into the internal iliac artery. A grid with 20-mm spaces was placed over the angiogram in the region of the medial thigh. The number of contrast-opacified arteries crossing over circles and the total number of lines encompassing the medial thigh area were counted in a blinded fashion. The angiographic score was calculated as the ratio of overlying opacified arteries divided by the total number of lines in the ischemic thigh. This angiographic score reflects vascular density in the medial thigh.

Intraarterial doppler guidewire measurement of blood flow

Blood flow was quantified in vivo before selective internal iliac angiography on New

Zealand White rabbits using a 0.018-inch Doppler guidewire (Cardiometrics, CA) (21). The wire was advanced via the 3-F infusion catheter positioned at the origin of the common iliac artery to the proximal segment of the iliac artery supplying the ischemic limb. APV (average peak velocity) was recorded at rest maximum. APV was recorded after bolus injection of 2 mg papaverine. After completing identical measurements in the normal limb, selective internal iliac angiography was performed as described above.

The angiographic luminal diameter of the internal iliac artery in the ischemic limb and of the external iliac artery in the normal limb were determined using an automated edge-detection system as previously described (21). The film selected for analysis was scanned with a high resolution video camera; the signal produced by the video camera was digitized and displayed on a video monitor (Laser Scan Image Comm, Santa Clara, CA). Center-lines were traced manually for a 10-mm long segment beginning immediately distal to the tip of the Doppler wire. The contents were subsequently detected automatically on the basis of the weighted sum of the first and second derivative functions applied to the digitized brightness information. The vascular diameter was then measured at the size of the Doppler sample volume (5mm distal to the wire top). Cross-sectional area was calculated assuming a circular lumen. Doppler-derived flow was calculated as previously described (21).

Measurement of blood pressure

Calf blood pressure was measured using Doppler Flowmeter. The pulse of the posterior tibial artery was identified using a Doppler probe, and the systolic blood pressure in both limbs was determined using standard techniques (21). The calf blood pressure ratio was defined for each rabbit as the ratio of systolic pressure of the ischemic limb to that of the normal limb.

Statistical analysis

All values are expressed as mean ± SEM. Analysis of variance with subsequent Duncan's test was used to determine the significance of differences in multiple comparisons. Differences P with

value less than 0.05 were considered significant.

Results

Angiogenesis induced by intra-muscular injection of "naked" human HGF plasmid in rat ischemia model

Initially, we measured rat endogenous HGF concentration in the hindlimb. As shown in Fig. 1a, HGF concentration in the ischemic hindlimb was significantly decreased as compared to that in non-ischemic hindlimb as control. Similar results were obtained from human blood vessels of patients with peripheral arterial disease (12). Down-regulation of the endogenous HGF system may be related to the pathogenesis of peripheral arterial disease, since HGF showed an anti-apoptotic action on endothelial cells, in addition to its mitogenic activity (22,23). The decrease in local HGF production in the ischemic hindlimb might be due to transforming growth factor-β or hypoxia, which are strong suppressors of vascular HGF in vitro as well as in vivo (24,25). Given the significant decrease in endogenous HGF production in the ischemic limb, we hypothesized that transfection of human HGF vector into the ischemic limb might result in a beneficial effect in hypoxia. Therefore, HGF plasmid was intra-muscularly transfected into the ischemic hindlimb of rats in which the femoral artery was excised to induce unilateral hind limb ischemia.

First, we transfected luciferase gene into the ischemic hindlimb, to examine the feasibility of gene transfer using naked plasmid DNA. Consistent with the previous reports (16), luciferase activity could be detected in the muscle transfected with luciferase vector (control: not detected, luciferase: 64400 ± 12600 RLU/ g tissue, $P<0.01$). Then, we measured human HGF concentration in the ischemic hindlimb transfected with human HGF or control vector. Expectedly, human immunoreactive HGF readily detected in the hindlimb transfected with human HGF vector, but not control vector at 4 days after transfection (Fig. 1b, $P<0.01$). Followed by increase in human HGF concentration, injection of human HGF vector into the ischemic hindlimb resulted in a significant increase in blood flow from 3 weeks after transfection to 5 weeks after transfection ($P<0.01$), as

shown in Figs. 2a & 2b. The degree of increase in blood flow by naked plasmid DNA was observed in a dose-dependent manner ($P<0.01$). Moreover, transfection of human HGF vector significantly increased capillary density as stained by alkaliphosphatase (a marker of endothelial cells) in ischemic hindlimb around the injection site as compared to hindlimb transfected with control vector (Fig. 3a & 3b, $P<0.01$). These results clearly demonstrated that transfection of human HGF vector into ischemic hindlimb induced therapeutic angiogenesis for the treatment of peripheral arterial disease. Moreover, we compared the effect of plasmid HGF DNA with recombinant HGF on the blood flow. Importantly, the increase in blood flow was much greater in rats transfected with 500 μg DNA than that administered with 25 or 50 μg of recombinant HGF at 5 weeks after transfection ($P<0.01$, Fig. 4). These results demonstrate that angiogenesis induced by gene transfer of HGF would be beneficial as compared to a single intra-arterial administration of rHGF.

Angiogenesis induced by intra-muscular injection of "naked" human HGF plasmid in rabbit ischemia model

Given the successful therapeutic angiogenesis using "naked" human HGF plasmid in rat ischemia model, the feasibility of gene therapy was also examined in a rabbit hindlimb ischemia model as pre-clinical study for human gene therapy. Therefore, naked human HGF plasmid was intra-muscularly injected into the ischemic hindlimb of rabbits in which the femoral artery was excised to induce unilateral hind limb ischemia. There was no significant difference in body weight between the rabbits treated with HGF plasmid and control vector on day 45 after surgery (data not shown). Intra-muscular injection of HGF plasmid into the ischemic limb on day 10 after surgery produced significant augmentation of collateral vessel development as assessed by angiography on day 31 and 45 in the ischemia model in a dose-dependent manner, as shown in Fig. 5 ($P<0.01$). Serial angiograms revealed progressive linear extension of collateral arteries from the origin stem artery to the distal point of the reconstituted parent vessel in HGF-treated animals (Fig. 5). Of importance, injection of HGF vector also reduced severe necrosis due to ischemia in rabbit muscle,

while vehicle-treated rabbits demonstrated severe necrosis of muscle (data not shown). Consistent with induction of angiogenesis, a significant increase in blood flow as assessed by doppler flow wire at basal condition was observed in the rats transfected with HGF plasmid as compared to rats transfected with control vector (Fig. 6, $P<0.01$). Papaverin treatment increased the blood flow both in rats transfected with HGF plasmid and control vector. Even under papaverin treatment, blood flow was significantly increased in rats transfected with HGF vector as compared to control vector (Fig. 6, $P<0.01$). A significant increase in the blood pressure ratio of ischemic limb to normal limb was also observed in the rats transfected with HGF plasmid as compared to control vector (the blood pressure ratio of ischemic limb to normal limb of rabbits transfected with control vector is 0.67 ± 0.07; Fig. 7, $P<0.01$).

Discussion

In patients with critical limb ischemia, since there is no pharmacological treatment. Amputation, despite its associated morbidity, mortality, and functional implications (1,26,27), is often recommended as a solution to the disabling symptoms, in particular excruciating ischemic rest pain (28-30). Indeed, a second major amputation is required in nearly 10 % of such patients. Consequently, the need for alternative treatment strategies in patients with critical limb ischemia is compelling. A novel therapeutic strategy using angiogenic growth factors to expedite and/or augment collateral artery development has recently entered the realm of treatment of ischemic diseases. Indeed, the clinical utility of gene therapy using VEGF gene has been recently reported for the treatment of critical limb ischemia and myocardial ischemia (4-6). The present study raises the possibility of a new strategy, therapeutic angiogenesis using HGF as gene therapy, instead of VEGF, for the treatment of patients with critical limb ischemia. HGF is a mesenchyme-derived pleiotropic factor which regulates cell growth, cell motility, and morphogenesis of various types of cells, and is thus considered a humoral mediator of epithelial-mesenchymal interactions responsible for morphogenic tissue interactions during embryonic development and organogenesis (31,32).

Based on our recent finding that HGF exclusively stimulated the growth of endothelial cells without VSMC replication, we further studied the capability of HGF to stimulate angiogenesis as means of gene therapy.

Although the feasibility of therapeutic angiogenesis using HGF has been reported in the experimental models (12), none of reports have examined the potential utility of HGF gene transfer to stimulate angiogenesis. Here, we demonstrated direct in vivo evidence of therapeutic angiogenesis induced by HGF gene transfer both in rat and rabbit ischemia models. Notably, a single intra-muscular injection of naked HGF plasmid was sufficient to induce therapeutic angiogenesis in the rat and rabbit hind limb ischemia model. Thus, in vivo transfer of HGF gene into the muscle opens up the possibility of local gene therapy for untreatable peripheral arterial diseases, although existing methods have many limitations such as low efficiency and/or potential toxicity.

What is the clinical relevance of therapeutic angiogenesis induced by HGF as compared to VEGF ? First, HGF may not cause edema as a side-effect, as it does not increase permeability, different from VEGF. Second, c-met has been reported to be up-regulated in response to hypoxia in a myocardial ischemia model (14), probably enhancing the angiogenic activity of HGF. Third, previous reports showed elevated levels of cell-associated matrix degrading enzymes (MMP-1 etc.) and enhanced plasmin-generating ability (uPA) by HGF (33-35). Interestingly, uPA activates pro-HGF in vitro, and activation of pro-HGF involves the formation of a stable complex between pro-HGF and uPA (30), suggesting that the biological effects of HGF can be titrated in vivo by the level of uPA activity. Increased amounts of uPA locally induced by HGF may condition the tissue microenvironment by rendering HGF bioavailable to its target cells. Fourth, HGF has been postulated to promote angiogenesis as a result of a combination of direct effects on endothelial cells and indirect effects, including paracrine up-regulation of VEGF, on VSMC (13). Fifth, decrease in endogenous vascular HGF may increase the threshold of safety against HGF, as the present and previous studies demonstrated the decrease in endogenous HGF concentration in ischemic condition (12,25).

One of the distinguishing features of HGF mentioned above the fact that the HGF gene encodes a secretary signal sequence-might be exploited as part of a strategy designed to accomplish therapeutic angiogenesis by arterial gene transfer. Previous reports mentioned that site-specific transfection of rabbit ear arteries with the plasmid encoding the gene for human growth hormone-a secreted protein-yields local levels of human growth hormone equivalent to what has been considered to be in a physiologic range-despite that fact that immunohistochemical examination of the transfected tissue disclosed evidence of successful transfection in <1% of cells in the transfected arterial segment (36). Thus, gene products that are secreted may have profound biological effects, even when the number of transduced cells remains low. In contrast for genes such as bFGF which do not encode a secretory signal sequence, transfection of a much larger cell population might be required for that intracellular gene product to express its biological effects. In these cases, higher efficient transfection method must be necessary.

Overall, the present studies suggest a novel therapeutic strategy that might reduce the symptoms of critical limb ischemia, utilizing the angiogenic properties of HGF gene transfer. Currently, we plan to start human clinical trial using HGF naked plasmid DNA to treat peripheral arterial disease. In addition, stimulation of new vessel formation by HGF is likely to create new therapeutic options in angiogenesis-dependent conditions such as wound healing, inflammatory diseases, ischemic heart disease, myocardial infarction and peripheral arterial disease.

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Figure Legends

Figure 1. (a) Muscular rat HGF concentration in non-ischemic and ischemic hindlimb.

Non-ischemic limb = muscle from non-ischemic rats, Ischemic limb = muscle from ischemic rats. Each group contains 7-8 animals.

(b) Human HGF concentration in muscle of ischemic hindlimb transfected with control or human HGF plasmid.

Control = muscle from ischemic hindlimb transfected with control vector (500 μg), plasmid (500 μg) = muscle from ischemic hindlimb transfected with human HGF vector (500 μg). N.D. = not detected. Each group contains 5-8 animals.

Figure 2. Effect of intra-muscular injection of human HGF plasmid in rat ischemic limb model (design 1)

(a) Typical Image of myocardial blood flow analyzed by laser Doppler imager at 5 weeks after transfection.

Control = right ischemic hindlimb transfected with control vector (500 μg), b) HGF plasmid = right ischemic hindlimb transfected with HGF vector (500 μg).

Intramuscular injection of plasmid was performed to right ischemic hindlimb. Panels show color-coded images representing blood flow distribution. Low or no perfusion is displayed as dark blue, whereas the highest perfusion is displayed as white.

(b) Quantitative analysis of blood flow in right hindlimb.

control = blood flow in ischemic hindlimb transfected with control vector (500 μg), HGF 100-500 μg = blood flow in ischemic hindlimb transfected with HGF vector (100 – 500 μg/body). pre = pretreatment, 1, 3, 5 = 1, 3 or 5 weeks after transfection.

##P<0.01 vs. Control. Each group contains 7-8 animals.

Figure 3. Effect of the transfection of HGF vector on the vascular formation.

(a) Representative cross-sections (200X): Control = muscle from rats transfected with control

vector (500 μg), HGF = muscle from rats transfected with human HGF vector (500 μg).

(b) Effect of the transfection of human HGF vector on the number of vessels.

Control = muscle from rats transfected with control vector (500 μg), HGF/100, 250, 500 = muscle from rats transfected with human HGF vector (100, 250 or 500 μg). Each group contains 7 to 8 animals.

Figure 4. Comparison of the increase in blood flow induced by HGF gene transfer and a single administration of recombinant HGF (design 2).

rHGF = rats treated with rHGF (25 or 50 μg/body), HGF = rats transfected with human HGF vector (500 μg/body). pre = pretreatment, 1, 3, 5 = 1, 3 or 5 weeks after transfection. *P<0.01 vs. pretreatment. Each group contains 7 to 8 animals.

Figure 5. Effect of the transfection of HGF vector on the vascular formation in rabbit hindlimb ischemic model

(a) Representative angiograms:

(A) Control = rabbit transfected with control vector (2 mg), (B) HGF = rabbit transfected with human HGF vector (2 mg).

(b) Effect of the transfection of HGF vector in rabbit hindlimb ischemic model on angiographic score expressed as percent change compared to vehicle.

control = rabbit transfected with control vector (2mg/body), HGF 1mg or 2 mg = rabbit transfected with human HGF vector (1 or 2 mg/body). **P<0.01 vs. control at corresponding time point. Control group contains 4 animals; each HGF group contains 3 animals.

Figure 6. Effect of transfection of human HGF plasmid on percent increase in blood flow as assessed by doppler flow wire.

control = rabbit transfected with control vector (2mg/body), HGF = rabbit transfected with human HGF vector (1 or 2 mg/body). Pre-BF = before treatment with papaverin, post-BF = after treatment

with papaverin. *P<0.05, **P<0.01 vs. control at corresponding time point. Control group contains 4 animals; each HGF group contains 3 animals.

Figure 7. Effect of transfection of human HGF plasmid on percent increase in the ratio of blood pressure in ischemic hindlimb to that in (contralateral) non-ischemic hindlimb.

control = rabbit transfected with control vector (2mg/body), HGF = rabbit transfected with human HGF vector (1 or 2mg/body). *P<0.05, **P<0.01 vs. control. Control group contains 4 animals; each HGF group contains 3 animals.

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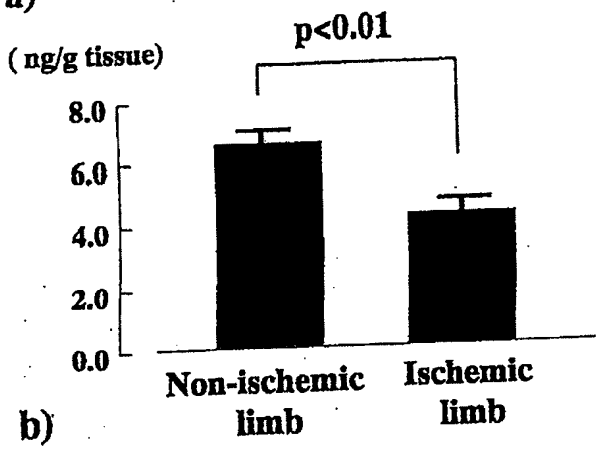
a)


p<0.01
(ng/g tissue)
8.0
6.0
4.0
2.0
0.0
Non-ischemic limb
Ischemic limb

b)

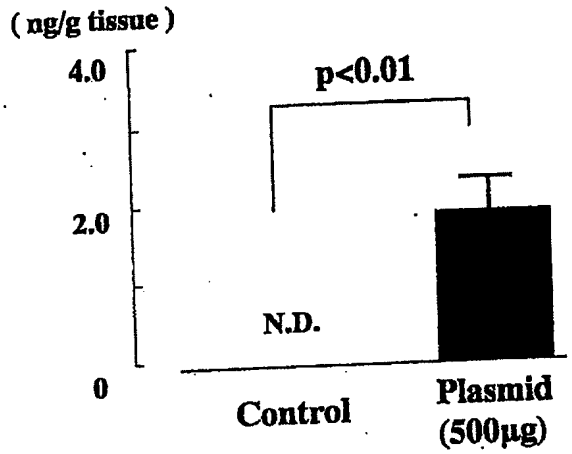

(ng/g tissue)
4.0
2.0
0
p<0.01
N.D.
Control
Plasmid (500µg)

Fig 1

Fig 2 (a)

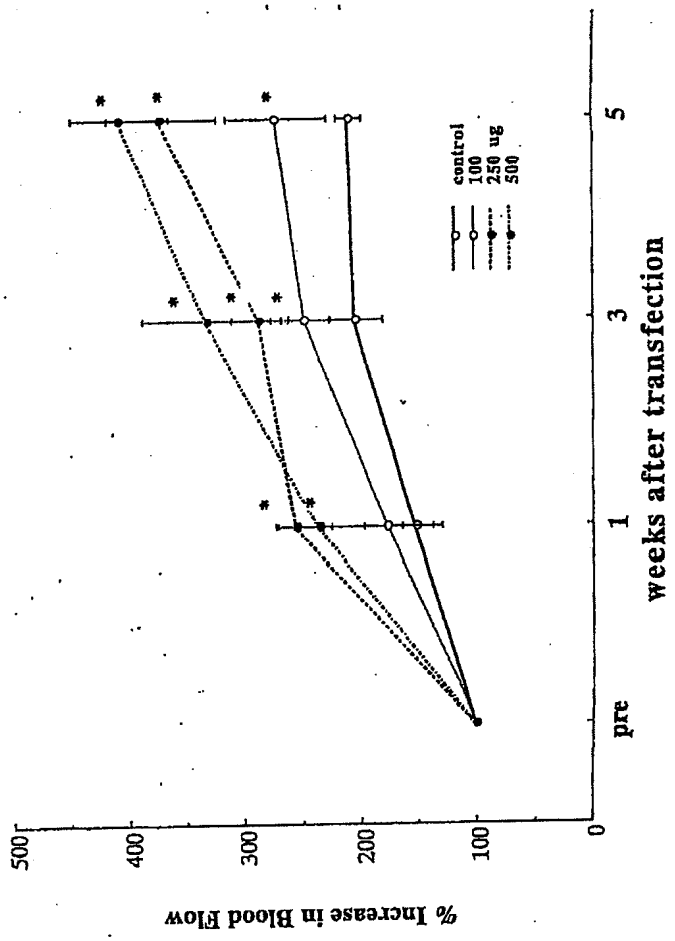

% Increase in Blood Flow
500
400
300
200
100
0
pre
1
3
5
weeks after transfection
control
100
250 ug
500

Fig 2 (b)

Fig 3

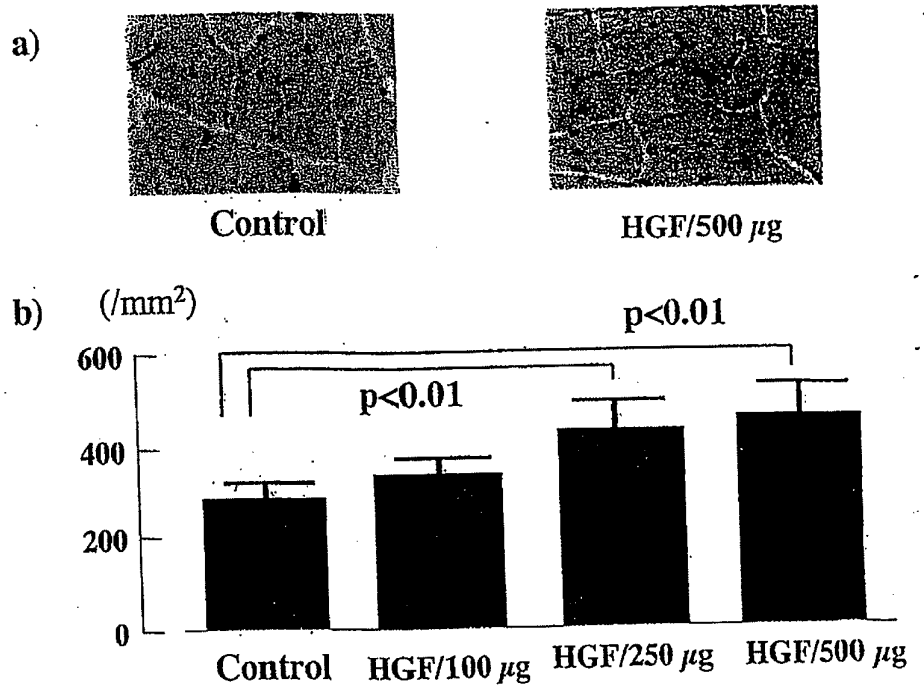

a)
Control
HGF/500 μg
b)
(/mm²)
p<0.01
p<0.01
600
400
200
0
Control
HGF/100 μg
HGF/250 μg
HGF/500 μg

Fig 4

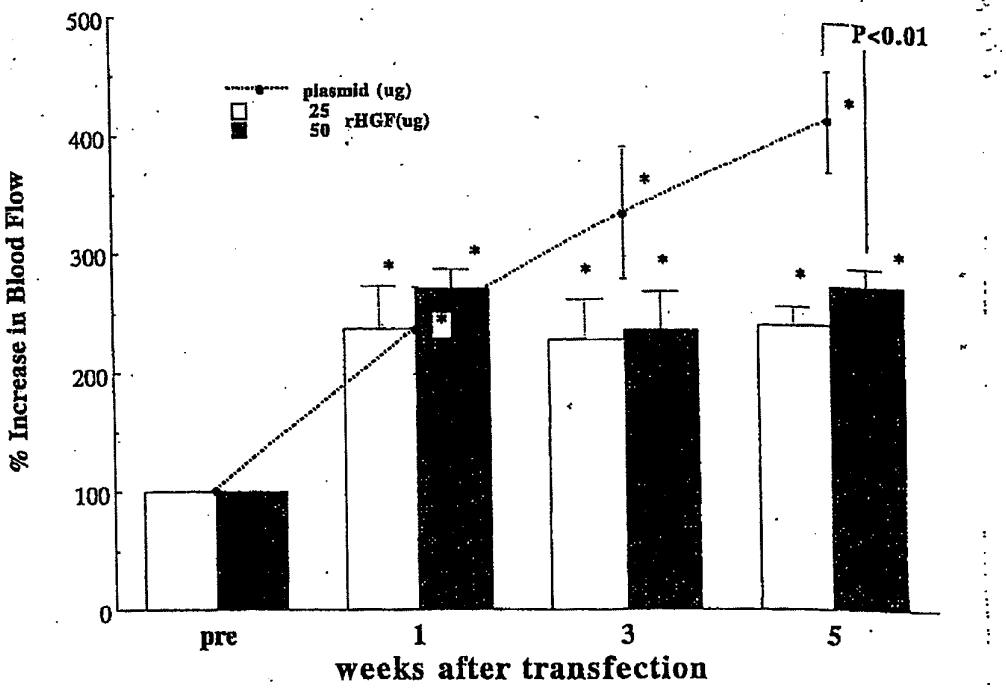

P<0.01
plasmid (ug)
25
50 rHGF(ug)
% Increase in Blood Flow
500
400
300
200
100
0
pre
1
3
5
weeks after transfection

Fig 5 (a)

% Increase in Angiographic Score
400
300
200
100
0
score (3W)
score (5W)
control
HGF 1mg
HGF 2mg
**
**

Fig 5 (b)

Fig 6

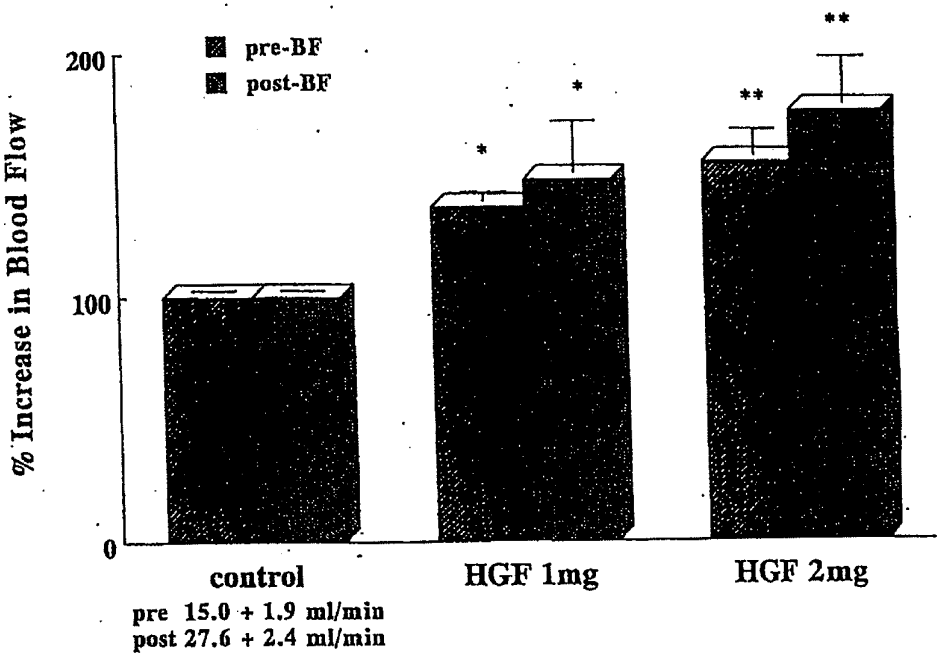
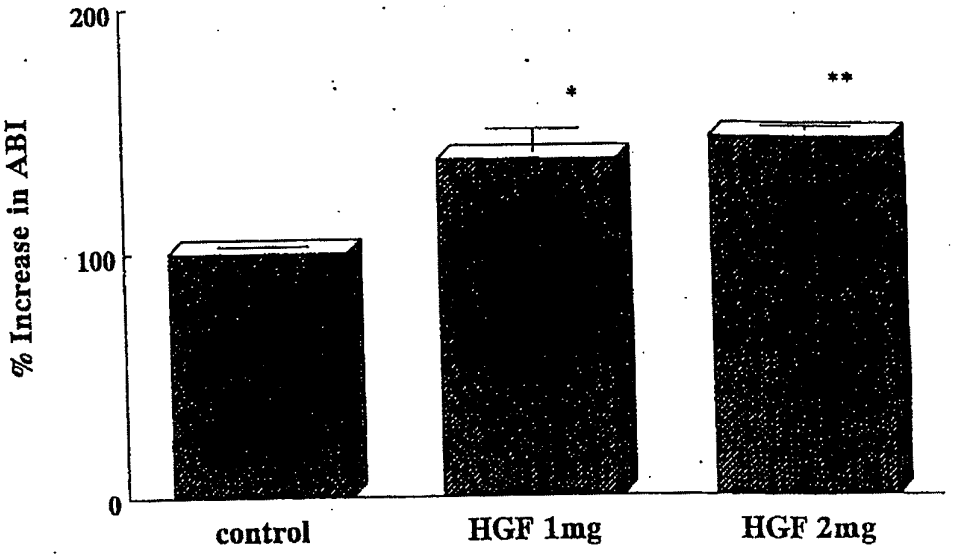

pre-BF
post-BF
200
100
0
% Increase in Blood Flow
*
*
**
**
control
HGF 1mg
HGF 2mg
pre 15.0 + 1.9 ml/min
post 27.6 + 2.4 ml/min

Fig. 7



Angiogenesis Induced by Hepatocyte Growth Factor in Non-infarcted Myocardium and Infarcted Myocardium: Up-regulation of Essential Transcription Factor for Angiogenesis, Ets

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Running title: Angiogenesis induced by HGF

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A list of abbreviations

HGF = hepatocyte Growth Factor

VEGF = Vascular Endothelial Growth Factor

HVJ = Hemagglutinating Virus of Japan

PCNA = Proliferating Cell Nuclear Antigen

MI = Myocardial Infarction

EIA = Enzyme Immuno-Assay

Abstract

Background: The feasibility of a novel therapeutic strategy using angiogenic growth factors to expedite and/or augment collateral artery development has recently entered the realm of treatment of ischemic diseases. Hepatocyte growth factor (HGF) is a novel member of endothelium-specific growth factors whose mitogenic activity on endothelial cells is very potent. Although it has been demonstrated that HGF is a potential angiogenic growth factor in in vitro culture systems, there is no direct in vivo evidence for the angiogenic activity of HGF in physiological conditions. In this study, we hypothesized that transfection of HGF gene into infarcted myocardium could induce angiogenesis, potentially resulting in a beneficial response to hypoxia.

Methods & Results: Human HGF gene or control vector driven by SRα promoter was transfected into rat myocardium by HVJ-liposome method. Four days after in vivo transfection of human HGF gene, there was a marked increase in human immunoreactive HGF as compared to control vector ($P<0.01$). In myocardium transfected with HGF vector, a significant increase in PCNA-positive endothelial cells was observed, while few PCNA-positive endothelial cells were detected in both control-vector-transfected and untreated myocardium. The number of vessels around the HGF injection sites was significantly increased as compared to control vector or vehicle ($P<0.01$). Angiogenic activity induced by the transfection of HGF vector was also confirmed by the activation of a transcription factor, ets, which is essential for angiogenesis, assessed the immunohistochemical study and the gel shift mobility assay. Furthermore, we studied the pathophysiological role of HGF in a myocardial infarction model. The concentration of endogenous HGF was significantly decreased in infarcted myocardium. Therefore, we hypothesized that transfection of HGF gene into infarcted myocardium could induce a beneficial response to the decreased endogenous HGF. Indeed, transfection of human HGF into infarcted myocardium also resulted in a significant increase in the number of vessels ($P<0.01$), accompanied by marked induction of ets binding activity and a significant increase in blood flow.

Conclusion: Overall, the present results provide direct in vivo evidence for the induction of angiogenesis by transfection of human HGF gene in rat non-infarcted and infarcted myocardium. The constant production of local HGF resulting from the transgene may be considered as an innovative therapeutic angiogenesis strategy for ischemic diseases such as myocardial infarction.

Introduction

Recent progress in molecular biology has led to the development of gene therapy [1-3] as a new strategy to treat a variety of cardiovascular diseases. Targeted disease range from single gene deficiency diseases to more complex diseases in adults such as restenosis after angioplasty. One obvious major target in the field of gene therapy is cardiac diseases, including myocardial infarction and cardiomyopathy. Recent reports have described that the administration of basic fibroblast growth factor (b-FGF) or vascular endothelial growth factor (VEGF) into the pericardium salvaged infarcted myocardium via formation of neovasculature [4,5]. The expression of angiogenic factors (as exemplified by basic and acidic fibroblast growth factor, TGF-β and vascular endothelial growth factor) due to gene transfer might induce neovascularization, **resulting in the salvage of the infarcted myocardium.** It is not surprising that gene transfer of angiogenic factors such as bFGF and VEGF may rescue the infarcted myocardium rather than neovascularization therapy with a recombinant. As we have previously reported successful gene transfer in a myocardial infarction model [6,7], we reasoned that transfection of angiogenic growth factor genes is a potential gene therapy for myocardial infarction.

We have focused on hepatocyte growth factor (HGF), which is a mesenchyme-derived pleiotropic factor that regulates cell growth, cell motility, and morphogenesis in various types of cells. Also HGF is considered a humoral mediator of epithelial-mesenchymal interactions responsible for morphogenic tissue interactions during the period of embryonic development and organogenesis [8,9]. Recently, HGF has been reported as a mitogen exclusively for endothelial cells without the replication of vascular smooth muscle cells (VSMC) [10-13], thereby indicating it as a potential angiogenic growth factor. Indeed, activation of the HGF system promoted angiogenesis in a Matrigel system [14], however, this system provides far from physiological conditions. Thus, there is no direct in vivo evidence of the angiogenic action of HGF. Moreover, HGF and its specific receptor, c-met, are expressed in the heart and blood vessels including endothelial cells and vascular smooth muscle cells [15]. In this study, we addressed two specific issues, to examine the feasibility of gene therapy for myocardial infarction: 1) whether over-expressed HGF in non-infarcted and infarcted myocardium induces neovascularization, and 2) what molecular mechanisms are involved in angiogenesis induced by over-expression of HGF.

Results

In vivo transfection of human HGF into non-infarcted myocardium

To examine the effect of HGF gene transfected into the myocardium, we first measured human immunoreactive HGF in non-infarcted myocardium transfected with human HGF vector or control vector at 4 days after transfection. As shown in Fig. 1a, human immunoreactive HGF was readily detected in myocardium transfected with human HGF vector, by EIA using specific human anti-HGF antibody that did not cross-react with rat HGF, while human HGF could not be detected in myocardium transfected with control vector. Similarly, human HGF could not be detected in untransfected myocardium. **Interestingly, rat endogenous immunoreactive HGF concentration was significantly increased in myocardium transfected with human HGF vector, as assessed by EIA using specific rat anti-HGF antibody at 4 days after transfection (Fig. 1b). These data suggest that endogenous cardiac HGF was induced by over-expressed exogenous HGF.**

Angiogenesis induced by human HGF gene transfer

Given the successful in vivo transfer of human HGF vector into the myocardium, the proliferation of endothelial cells was measured at 4 days after transfection by immunohistochemical analysis using PCNA staining, to determine the mitogenic action of locally produced human HGF. The transfection of human HGF vector significantly increased PCNA-stained endothelial cells (a marker of DNA synthesis) in the myocardium around the injection site as compared to myocardium transfected with control vector (**Fig. 2a**). The PCNA-positive cells were identified as endothelial cells, but not fibroblasts or cardiac myocytes, as assessed by HE staining and endothelium-specific staining with endothelial cell specific antibody (RECA). In intact normal hearts, less than 1% of endothelial cells in the myocardium stained positively for PCNA by immunohistochemical technique (data not shown). As shown in **Fig. 2b,** the number of PCNA-positive stained endothelial cells was significantly increased in myocardium transfected with human HGF vector as compared to control vector-transfected myocardium and untransfected myocardium ($P<0.01$).

Next, we measured the number of vessels at 14 days after transfection. Of importance, the number of vessels was significantly increased in myocardium transfected with HGF vector as compared to control vector ($P<0.01$), as shown in **Figs. 3a & 3b.** However, for angiogenesis to

have therapeutic feasibility, the size of neovascularized vessels is important. Therefore, we also counted the number of vessels, according to the size. Microvessels less than 10 μm were significantly increased in myocardium transfected with HGF gene (**Fig. 3b**). In addition to microvessels, vessels more than 10 μm were also significantly increased in myocardium transfected with HGF vector, as shown in **Fig. 3b**. These results indicate that the angiogenesis induced by HGF could produce small-to-medium sized vessels as well as micro-capillary vessels.

Up-regulation of essential transcription factor for angiogenesis, ets, by HGF

To further confirm the induction of angiogenesis by in vivo transfection of HGF gene, an essential transcription factor for angiogenesis, ets-1, was analyzed. Transfection of HGF vector caused a large increase in ets activity in myocardium transfected with HGF vector, which was assessed by gel mobility shift assay (**Fig. 4**). Moreover, complete competition for the increased binding of ets-1 by an excess amount of cold ets-1 probe was observed (**Fig. 4a**). Densitometric analysis revealed a significant increase in ets-1 binding activity in myocardium transfected with HGF vector as compared to control vector ($P<0.01$, **Fig. 4b**). To investigate the localization of up-regulated ets-1 binding activity, we also performed immunohistochemical analysis. Immunohistochemical studies showed little staining in myocardium transfected with control vector and untransfected myocardium. In contrast, in myocardium transfected with human HGF vector, positive staining of ets-1 was observed in endothelial cells and VSMC around neovascularized vessels (**Fig. 5**).

Induction of angiogenesis by over-expressed HGF in myocardial infarction model

Given the strong angiogenic activity of the over-expression of human HGF, we further studied the physiological role of over-expressed HGF in a myocardial infarction model. Thus, we measured the endogenous cardiac HGF level in infarcted myocardium. Of importance, cardiac immunoreactive rat HGF was significantly decreased at 14 days after myocardial infarction ($P<0.01$, **Fig. 6b**), as compared to that in sham-operated rat. Therefore, we concluded that transfection of HGF gene into the infarcted myocardium could induce a beneficial response to the decreased endogenous HGF. **Importantly, the expression of transfected HGF was sustained at least up to 14 days after transfection (Fig. 6a). Interestingly, transfection of human HGF**

vector significantly increased rat endogenous cardiac HGF (Fig. 6b). Moreover, a significant increase in the number of vessels was observed in the myocardium transfected with human HGF vector as compared to that in control vector at 14 days after transfection (P<0.01, Fig. 7), accompanied by induction of ets binding activity (Fig. 8). Similar to the experiments in non-infarcted myocardium, microvessels less than 10 μm were significantly increased in myocardium transfected with HGF gene. In addition to microvessels, vessels more than 10 μm were also significantly increased in myocardium transfected with HGF vector (**Fig. 7b**). Moreover, a marked increase in blood flow was observed using a laser Doppler perfusion imager in infarcted myocardium transfected with HGF vector (**Fig. 9**). Although blood flow in infarcted myocardium was markedly decreased as compared to non-infarcted myocardium, the over-expression of HGF significantly attenuated the decrease in blood flow in infarcted myocardium (**Fig. 10**, P<0.05).

Discussion

HGF has been postulated to be a potent angiogenic growth factor, since it is a mitogen for various endothelial cells and is also a potent inducer of tube formation [10-13]. Indeed, the angiogenic response to HGF has been reported in an experimental model of implanted reconstituted basement membrane (Matrigel) [14]. However, no report has described the angiogenic activity of HGF in physiological conditions in a normal tissue without any supplement such as Matrigel, and in addition, there are controversial reports that HGF was unable to stimulate angiogenesis [16]. Here, we demonstrated direct in vivo evidence of angiogenesis induced by HGF in non-infarcted and infarcted myocardium. The specificity of the angiogenic effect of in vivo transfection of HGF gene into the myocardium is supported by several lines of evidence: 1) Human HGF protein was increased by the transfection of human HGF vector. 2) An increase in the number of vessels and PCNA-positive stained endothelial cells was observed in myocardium transfected with HGF vector. 3) Induction of an essential transcription factor for angiogenesis, ets-1, by transfection of HGF vector was observed. More importantly, the current study demonstrated a significant increase in blood flow by the over-expression of HGF even in infarcted myocardium. These data clearly reveal

the potential utility of HGF gene transfer for the treatment of ischemic heart disease.

Members of the ets family play important roles in regulating gene expression in response to multiple developmental and mitogenic signals [17-20]. The ets family of transcription factors has a DNA-binding domain in common that binds to a core GGA(A/T) DNA sequence [21,22]. In situ hybridization studies have shown that the proto-oncogene c-ets is expressed in endothelial cells at the beginning of blood vessel formation, under normal and pathological conditions [23,24]. Thus, previous reports suggest that the ets family may activate the transcription of genes encoding collagenase 1, stromelysine 1 and urokinase plasminogen activator, which are proteases involved in extracellular matrix degradation [25-27]. It is believed that the ets family takes part in regulating angiogenesis by controlling the transcription of these genes whose activity is necessary for the migration of endothelial cells from pre-existing capillaries. In this study, we also demonstrated the up-regulation of endogenous HGF expression by exogenously transfected HGF gene, consistent with our previous findings in in vitro experiments [28]. Induction of ets activity by over-expressed HGF may regulate this auto-loop up-regulation of HGF, since the promoter region of the HGF gene contains a number of putative regulatory elements, such as a B cell- and macrophage-specific transcription factor binding site (PU.1/ETS), besides a interleukin-6 response element (IL-6 RE), a TGF-beta inhibitory element (TIE), and a cAMP response element (CRE) [29].

VEGF, another endothelium-specific growth factor, has been reported to be up regulated by hypoxia in vitro and by ischemia in vivo [30-32]. Therefore, VEGF is a likely mediator in the natural process of ischemia-induced myocardial neovascularization. In the clinical setting, the VEGF induced as a potent angiogenesis factor may not be sufficient to stimulate coronary collateral formation, therefore, administration of recombinant VEGF or VEGF gene to promote angiogenesis will be useful as a treatment for arteriosclerosis obliterans (ASO) and myocardial infarction in animal models and clinical patients [5,33,34].

On the other hand, the present data demonstrated that endogenously expressed HGF was markedly decreased in infarcted myocardium in the late phase (14 days), although the up-regulation of c-met has been previously reported in a myocardial infarction model in the acute phase [35]. Therefore, a sufficient supply of HGF by in vivo transfer of HGF gene into the infarcted myocardium would also enhance collateral formation. Of importance, the over-expression of HGF also has the ability to induce angiogenesis in a myocardial infarction model, in addition to non-

infarcted myocardium. Taken together, over-expression of HGF might enhance angiogenesis in myocardial infarction. Since the size of myocardial infarction correlates closely with mortality and residual impairment of function in survivors [36], a therapy targeted at promoting collateral formation would decrease the size of chronic myocardial infarction. **Indeed, the beneficial effect of HGF gene transfer was also reported by Ueda et al [37]. They demonstrated that transfection of HGF gene into transplanted hearts resulted in the attenuation of reperfusion injury [37]. In addition, we have previously reported anti-apoptotic action of HGF in endothelial cells [38]. Although it is likely that gene transfer of HGF may also work as anti-apoptotic factor in myocardial infarction model, further studies are necessary.**

The current studies suggest a novel therapeutic strategy that might reduce the extent of myocardial infarction, utilizing in vivo transfer of HGF gene, through the induction of coordinated transactivating gene, i.e., ets-1, which is necessary for angiogenesis. In this study, we employed direct injection into the non-infarcted and infarcted myocardium, since we have previously reported no evidence of cytotoxicity or inflammation caused by HVJ-liposome complex itself [6,7, 39]. Moreover, we have established an efficient in vivo gene transfer method into the heart using HVJ-liposome method via direct infusion into a coronary artery as well as incubation within the pericardium, without any damage to the myocardium [6]. The widespread transgene expression obtained by these approaches suggests that neovascularization induced by the over-expression of HGF gene may be useful for the treatment of ischemic heart disease.

In conclusion, manipulation of the formation of new vessels by means of the over-expression of HGF is likely to provide new therapeutic options in angiogenesis-dependent conditions such as wound healing, inflammatory diseases, peripheral vascular disease and ischemic heart disease such as myocardial infarction.

Materials and Methods

Construction of plasmids

To produce a HGF expression vector, human HGF cDNA (2.2 kb) was inserted into the Eco RI and Not I sites of pUC-SRα expression vector plasmid. In this plasmid, transcription of HGF cDNA was under the control of the SRα promoter [40,41].

Preparation of HVJ-liposome

We have previously reported a high efficacy of transfection with Hemagglutinating Virus of Japan (HVJ)-coated liposomes [6,7,39,42,43]. Briefly, phosphatidylserine, phosphatidylcholine, and cholesterol were mixed at a weight ratio of 1:4.8:2 in tetrahydrofuran. The lipid mixture (10 mg) was deposited on the sides of a flask by removal of the solvent in a rotary evaporator. The high mobility group (HMG)-1 purified from calf thymus was mixed with plasmid DNA (300 μg) in 200 μl balanced salt solution (BSS; 137 mM NaCl, 5.4 mM KCl, 10 mM Tris-HCl, pH 7.6) at 20°C for 1 hour, and then the mixture was added to the dried lipid. Liposome-DNA-HMG 1 complex suspension was mixed by vortex, sonication for 3 seconds, and shaking for 30 min. Purified HVJ (Z strain) was inactivated by UV irradiation (110 erg/mm^2/sec) for 3 min immediately prior to use. The liposome suspension (0.5 ml, containing 10 mg lipid) was mixed with HVJ (20,000 hemagglutinating units) in a total volume of 4 ml BSS. The mixture was incubated at 4°C for 10 min and then for 30 min with gentle shaking at 37°C. Free HVJ was removed from the HVJ-liposomes by sucrose density gradient centrifugation. The top layer of the sucrose gradient containing the HVJ-liposome-DNA complex was collected and used immediately.

In vivo gene transfer using direct injection approach

Sprague-Dawley rats (400-500 g; Charles River Breeding Laboratories) were anesthetized with an intraperitoneal injection of sodium pentobarbital (0.1 ml/100 mg). Rats were intubated and connected to a respirator. HVJ-liposome complex containing human HGF or control vector (10 μg/ml in liposomes) was injected directly into the apex of the non-infarcted heart with a 30G needle through a left lateral thoracotomy. Then 10 μl (0.1 μg plasmid) HVJ-liposome complex was injected in to each rat [6,7,39].

Measurement of HGF concentration in myocardium

To document successful transfection of HGF vector into the myocardium, we examined the production of human immunoreactive HGF [15,44]. Four days after the transfection, the hearts of the rats transfected with HGF or control vector were promptly taken away with removing excess fat after perfusion via the apex with saline, frozen in liquid nitrogen, and then stored at -70 °C. On the

day of the extraction, the tissue was thawed at 4 °C, weighed, and homogenized by polytron in an assay solution. Each specimen was centrifuged at 20,000 X g for 30 minutes at 4 °C, to remove the lysates. The concentration of HGF in the myocardium was determined by the enzyme-immunoassay with an anti-human HGF antibody, as described previously [11,15]. Briefly, a rabbit anti-rat or an anti-human HGF IgG was coated on 96-well plates (Corning, NY) at 4°C for 15 hours. After blocking with 3% bovine serum albumin in phosphate-buffered saline (PBS), the conditioned medium was added to each well. Afterwards, the preparation was incubated for 2 hours at 25 °C. Wells were washed three times with PBS containing 0.025% Tween 20 (PBS-Tween). A biotinylated rabbit anti-human HGF IgG was added and the preparation was incubated for 2 hours at 25 °C. After the wash with PBS-Tween, wells were incubated with a horseradish peroxidase-conjugated streptavidin-biotin complex in PBS-Tween. The enzyme reaction was initiated by adding substrate solution composed of 2.5 mg/ml *o*-phenylenediamine, 100 mM sodium phosphate, 50 mM citric acid, and 0.015% H_2O_2. The enzyme reaction was suspended by adding 1 M H_2SO_4, and the absorbance was measured at 490 nm of wavelength. The antibody against human HGF reacts with only human HGF, not with rat HGF [44].

Immunohistochemical studies

Monoclonal antibodies against endothelial specific RECA (1A4, DAKO, Glostup, Denmark) and proliferating cell nuclear antigen (PCNA) (PC-10, DAKO) were used as specific markers for endothelial cells and proliferating cells, respectively, in paraffin-embedded sections. Also, anti-ets-1 (Santa Cruz, CA) antibody was used to analyze the expression of ets-1 induced by the transfection of HGF gene. Immunohistochemical staining was performed using the immunoperoxidase avidin-biotin complex system with nickel chloride (NiCl) color modification as previously described [45]. Briefly, 5-um sections were deparaffinized, rehydrated before blocking the endogenous peroxidase activity with 3% hydrogen peroxidase, and preincubated with 5% normal rabbit serum in Tris-HCl buffered saline (TBS) for 20 minutes. Diluted primary antibodies (1A4 1:50, PC-10 1:500, anti-ets-1 1:20) were then applied to the sections, and these sections were incubated for 30 minutes. With intervening washing in TBS, they were serially incubated with a 1:400 dilution of biotinylated rabbit, anti-mouse IgG (DAKO) in TBS for 30 minutes; streptavidin-biotinylated horseradish peroxidase complex (DAKO), diluted 1:100 in TBS for 30 minutes; 0.05%

3,3'-diaminobenzidine (DAB, Sigma Chemicals, St. Louis, MO) in 200 ml TBS to which had been added 0.2 ml 30% hydrogen peroxide and 1.0 ml 8% NiCl solution for 5 minutes. Sections were counter-stained with methylgreen, dehydrated in a graded series of alcohol concentrations, permeated with xylene, and then covered with a coverslip.

To analyze the number of vessels in the non-infarcted and the infarcted myocardium transfected with HGF vector or control vector, HE staining was performed. Two weeks after the transfection, rats were sacrificed and the hearts were removed after perfusion-fixation (110 mmHg) with 10% buffered formalin. **Three individual** sections from the middle of the transfected myocardium were analyzed. The number of vessels was counted under a light microscopy (magnification, x 100) in a blinded manner, after a selective staining with hematoxylin-eosin in a standard manner. The total number of vascules in each section was summed and expressed as number per section. At least ten individual sections were evaluated on each heart. **The areas, in which the number of vascules was quantified, were randomly selected in the injected site and around the injected site**. The animals were coded so that the analysis was performed without any knowledge of which treatment for each individual had received. The reproducibility of the results was assessed. Intraobserver variability was determined from triplicate measurements performed by one observer for all sections. The mean ± SD difference among measurements made by the same observer was 2.8 ± 0.5 %. Interobserver variability was determined from measurements of 10 randomly selected sections performed by the second observer in addition to the first one. The numerical difference between the measurements made by two observers was 3.1 ± 0.6 %. These observers were blinded to other data concerning the rats, as well as to the results of other observers.

Gel mobility shift assay

Rats were sacrificed at 1 week after the transfection, and the nuclear extracts were prepared from the non-infarcted and the infarcted myocardium transfected with HGF vector or control vector, as previously described [44,45]. In brief, rat hearts were homogenized with a Potte-Elvehjem homogenizer in 4 volumes of ice-cold homogenization buffer (10 mM Hepes [pH 7.5], 0.5 M sucrose, 0.5 mM spermidine, 0.15 mM spermin, 5 mM EDTA, 0.25 M EGTA, 7 mM beta-mercaptoethanol and 1 mM phenylmethylsulfonyl-fluoride). After the centrifugation at 12,000 x g for 30 min at 4 °C, each pellet was lysed in 1 volume of ice-cold homogenization buffer containing

0.1 % NP-40 by homogenizing in a Dounce homogenizer. Then it was centrifuged at 12,000 x g for 30 min at 4 °C and the pellet nucleus was washed twice with ice-cold buffer containing 0.35 M sucrose. After washing, the nucleus was pre-extracted with 1 volume of ice-cold homogenization buffer containing 0.05 M NaCl and 10 % glycerol for 15 min at 4 °C. The nucleus was then extracted with homogenization buffer containing 0.3 M NaCl and 10 % glycerol for 1 hr at 4 °C. Subsequently, the concentration of DNA was adjusted to 1 mg/ml. After pelleting the extracted nucleus at 12,000 x g for 30 min at 4 °C, 45 % $(NH4)_2SO_4$ was added to the supernatant. Then, the mixture was stirred for 30 min at 4 °C. The precipitated protein was collected at 17,000 x g for 30 min, resuspended in homogenization buffer containing 0.35 M sucrose, and stored in aliquots at -70 °C.

ODNs containing ets binding sites (5'-GTGCCGGGGTAGGAAGTGGGCTGGG-3'; only sense strands are shown) and mutated ets binding sites (5'-GTGCCGGGGTAccttGTGGGCTGGG-3'; underline showed mutated sequences) were labeled as a primer at the 3' end by 3' end-labeling kit (Clontech Inc., Palo Alto, CA). After end-labeling, ^{32}P-labeled ODNs were purified by the application to Nick column (Pharmacia). The binding mixtures (10 ul) including ^{32}P-labeled primers (0.5 - 1 ng, 10,000-15,000 cpm) and 1 ug polydeoxyinosinic-deoxycytidic acid (Sigma Co.) were incubated with 10 ug nuclear extract for 30 min at room temperature and then loaded onto 5 % polyacrylamide gel. The gels were subjected to electrophoresis, dried, and pre-incubated with parallel samples for 10 min before the addition of the labeled probe. As a control, samples were incubated with an excess (50 x, 100 x) of nonlabeled ets-1 ODN which completely abolished binding. Gels were analyzed by autoradiography.

In vivo gene transfer using a direct injection approach to a myocardial infarction model

For a myocardial infarction model, the left coronary artery (LCA) was ligated within 2 to 3 mm of the proximal aorta [6,46]. Ventricular premature beats, ventricular tachycardia, and ventricular fibrillation occurred after ligation and ST segment elevation was also detected on the ECG. All examined animals had a normal P-QRS coupling and exhibited normal sinus rhythm with an anesthetized heart rate of approximately 400 beats per min. Recanalization and reperfusion were not performed. Immediately after ligation, HVJ-liposome complex containing either human HGF or control vector (10 μg/ml in liposomes) was directly injected into the infarcted area with a 30G

needle through a left lateral thoracotomy. The volume of HVJ-liposome injected into the rats was 10 μl (0.1 ug plasmid) [6,7,39]. The rats were killed at either 3hours or 14 days after the ligation for the analyses of RT-PCR, EIA, and HE staining.

Monitoring cardiac blood flow

The measurement of blood flow with Laser Doppler Imager (LDI) has been previously described [48,49]. As it was clearly demonstrated that Laser Doppler flow velocity well correlates with the capillary density [48,49], we measured the cardiac blood flow by means of a Laser Doppler blood flowmeter (Laser Doppler Image, Moor Instruments, England). Indeed, we confirmed that the blood flow measured by LDI was well correlated with the capillary density (data not shown). LDI uses a 12-mW helium-neon laser beam that sequentially scans a 5X5 cm surface area with extremely high speed to be able to measure the blood flow in the myocardium. The blood flow at 1 mm under the surface can be measured. While scanning, blood cells that are in motion shift as the frequency of projection light according to the Doppler principle. Upon the termination of scanning, a color-coded image representing blood flow distribution is displayed on a monitor. The perfusion signal is subdivided into 14 different intervals, and each interval is displayed in a separate color. Low or no perfusion is displayed as dark blue, whereas the highest perfusion intervals is displayed as white. The stored perfusion values behind the color-coded pixels remain available for the data analysis. Before measuring, rats were anesthetized, intubated and connected to a respirator. Then, the chest was widely opened through a left lateral thoracotomy to scan directly the surface of the hearts. Perfusion analyses were performed sequentially; a) the non-infarcted myocardium transfected with control vector, b) the non-infarcted myocardium transfected with HGF vector, c) the infarcted myocardium transfected with control vector, and d) the infarcted myocardium transfected with HGF vector at 14 days after the ligation and the transfection. These laser images were quantitatively converted into histograms that represented the amount of blood flow on the x-axis and the number of pixels on the y-axis in the traced area. The average blood flow in each histogram was calculated to be evaluated.

Statistical analysis

All values are expressed as mean ± SEM. The analysis of a variance with a subsequent

Duncan's test was used to determine the significance of differences in the multiple comparisons. The differences with values of less than 0.05 were considered significant.

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Figure Legends

Figure 1. (a) Human immunoreactive HGF in the non-infarcted myocardium untransfected or transfected with human HGF vector or control vector at 4 days after the transfection.

Untransfected = the myocardium from untransfected rats, Control = the myocardium from rats transfected with control vector, HGF = the myocardium from rats transfected with human HGF vector.

N.D. = not detected, N = 8 per group.

(b) Measurement of rat cardiac HGF concentration in the non-infarcted myocardium transfected with human HGF vector or control vector at 4 days after the transfection.

Untransfected = the myocardium from untransfected rats, Control = the myocardium from rats transfected with control vector, HGF = the myocardium from rats transfected with human HGF vector. N = 8 per group.

Figure 2. **(a) Immunohistochemical staining of PCNA in the myocardium transfected with HGF or control vector.**

Representative cross-sections stained with anti-PCNA antibody: HGF = the myocardium from rats transfected with human HGF vector, Control = the myocardium from rats transfected with control vector. (200 X)

Arrows indicate PCNA positive endothelial cells.

(b) Effect of the transfection of human HGF vector on PCNA index (PCNA-positive endothelial cells) at 4 days after transfection.

Untransfected = the myocardium from untransfected rats, Control = the myocardium from rats transfected with control vector, HGF = the myocardium from rats transfected with human HGF vector. Each group contains 7 to 8 animals.

Figure 3. Effect of the transfection of HGF vector on the vascular formation.

(a) Representative cross-sections: HGF = the myocardium from rats transfected with human HGF vector (200X, 400X), Control = the myocardium from rats transfected with control vector (**200X, 400X**).

Long arrows indicate vessels more than 10 μm. Short arrows indicate vessels less than 10 μm.

(b) Effect of the transfection of human HGF vector on the number of vessels more than 10 μm (>10), vessels less than 10 μm (< 10) and the total number of vessels (total).

Control = the myocardium from rats transfected with control vector, HGF = the myocardium from rats transfected with human HGF vector.

##P<0.01 vs. Control. Each group contains 7 to 8 animals.

Figure 4. (a) Gel-mobility shift assay for ets binding site. N = P^{32}-labeled ODN containing ets-binding site without any nuclear extract, control = nuclear extracts (30 ug) from the myocardium transfected with control vector incubated with P^{32}-labeled ets probe for 30 minutes at room temperature without any competitor, HGF = nuclear extracts (30 ug) from the myocardium transfected with human HGF vector incubated with P^{32}-labeled ets probe, mis = non-labeled mismatched probe (x 50, x 100 excess), ets = non-labeled et probe (x 50, x 100 excess).

(b) Ets binding activity assessed by densitometry.

Control = the myocardium transfected with control vector, HGF = the myocardium transfected with human HGF vector. Each group contains 7 to 8 animals.

Figure 5. Immunohistochemical staining of ets-1 protein in the myocardium transfected with control or HGF vector.

Representative cross-sections stained with anti-ets-1 antibody:

HGF = the myocardium from rats transfected with human HGF vector, Control = the myocardium from rats transfected with control vector (400 X).

Arrows indicate ets-1 positive endothelial cells.

Figure 6. **(a) Measurement of human immunoreactive HGF (a) and rat cardiac HGF concentration (b) in the non-infarcted myocardium untransfected or transfected with human HGF vector or control vector at 14 days after the transfection.**

Sham = the myocardium without ligation, MI = the myocardium with ligation, MI+Control = the myocardium from infarcted rats transfected with control vector, MI+HGF = the myocardium from infarcted rats transfected with human HGF vector.

N.D. = not detected, N = 8 per group.

Figure 7. Effect of the transfection of HGF vector on the vascular formation in a myocardial infarction model.

a) Representative cross-sections: HGF (+) = the infarcted myocardium from rats transfected with human HGF vector (200X), HGF (-) = the infarcted myocardium from rats transfected with control vector (200X).

Long arrows indicate vessels more than 10 μm. Short arrows indicate vessels less than 10 μm.

(b) Effect of the transfection of human HGF vector on the number of vessels more than 10 μm, the vessels less than 10 μm and the total vessels.

Control = the myocardium from rats transfected with control vector, HGF = the myocardium from rats transfected with human HGF vector.

##P<0.01 vs. Control. Each group contains 7 to 8 animals.

Figure 8. Gel-mobility shift assay for ets binding site in the infarcted hearts.

MI= nuclear extracts (20 μg) from the myocardium transfected with control vector at 14 days after ligation and transfection with P^{32}-labeled ets probe, MI+HGF= nuclear extracts (20 μg) from the myocardium transfected with HGF vector at 14 days after the ligation and transfection with P^{32}-labeled ets probe, MI+HGF+cold probe= nuclear extracts (20 μg) from the myocardium transfected with HGF vector at 14 days after ligation and transfection with P^{32}-labeled ets probe and an excess amount of non-labeled ets probe(competitor)(x 50).

Figure 9. Typical Image and histogram of myocardial blood flow analyzed by laser Doppler imager.

a) HGF (-) In MI = Infarcted myocardium transfected with control vector, b) HGF (+) In MI = Infarcted myocardium transfected with HGF vector.

Left panels show color-coded images representing blood flow distribution. Low or no perfusion is displayed as dark blue, whereas the highest perfusion is displayed as white. Right panels demonstrate typical histograms, with blood flow was converted to x-axis and number of pixels was converted to y-axis.

Figure 10. Quantitative analysis of myocardial blood flow.

Untransfected = blood flow in untransfected heart, Control = blood flow in non-infarcted heart transfected with control vector, HGF = blood flow in non-infarcted heart transfected with HGF vector, MI = blood flow in infarcted heart transfected with control vector, MI+HGF = blood flow in infarcted heart transfected with HGF vector.

##P<0.01 vs. Untransfected and Control. Each group contains 4 animals.

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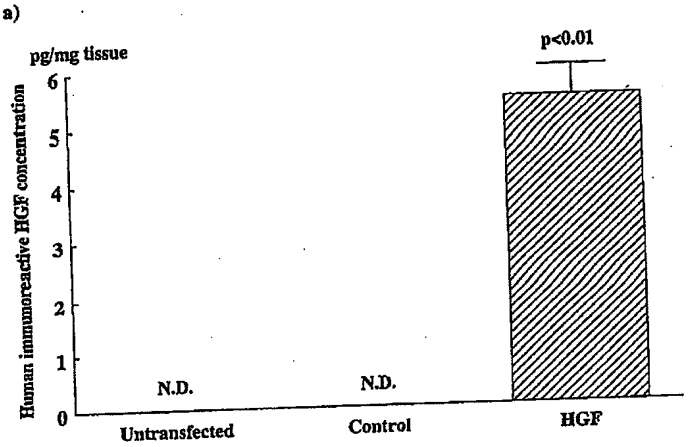
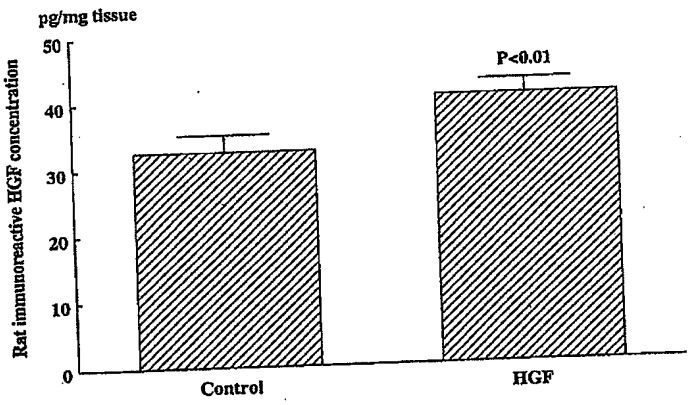
a)

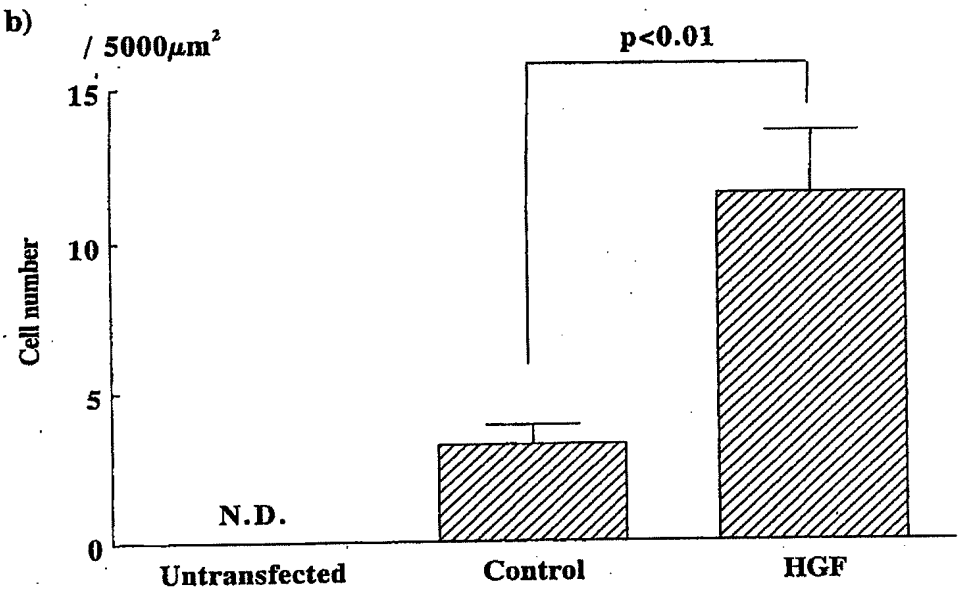

pg/mg tissue
Human immunoreactive HGF concentration
6
5
4
3
2
1
0
p<0.01
N.D.
N.D.
Untransfected
Control
HGF

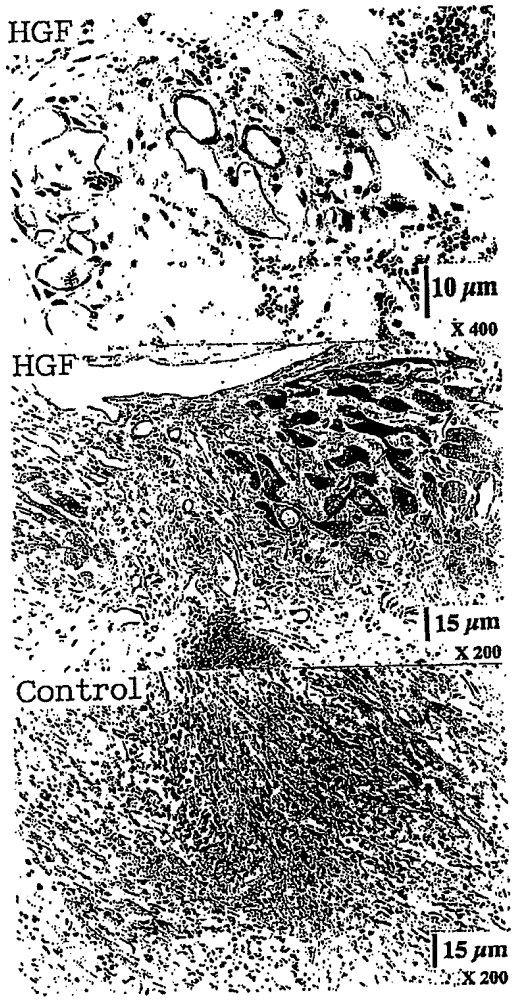
b)

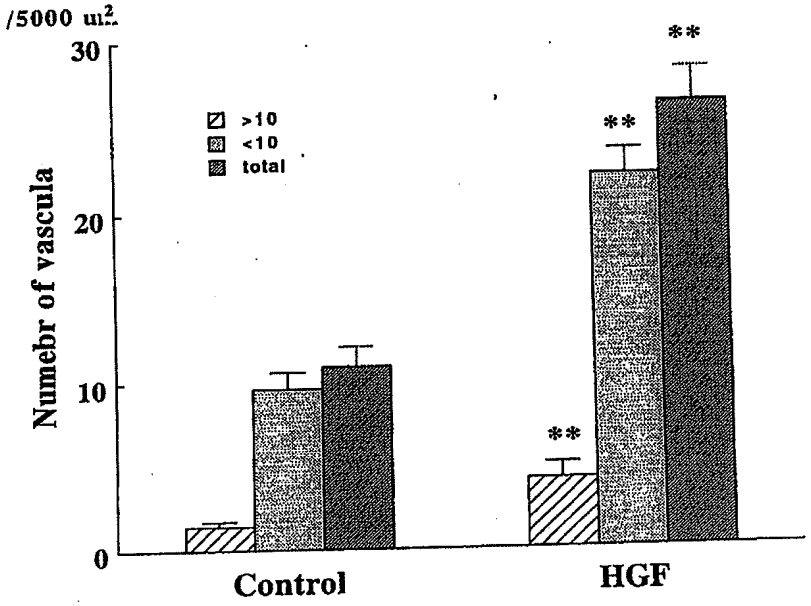

pg/mg tissue
Rat immunoreactive HGF concentration
50
40
30
20
10
0
P<0.01
Control
HGF


HGF


Control


b)
/ 5000μm²
p<0.01
15
10
5
0
Cell number
N.D.
Untransfected
Control
HGF


HGF
10 μm
X 400
HGF
15 μm
X 200
Control
15 μm
X 200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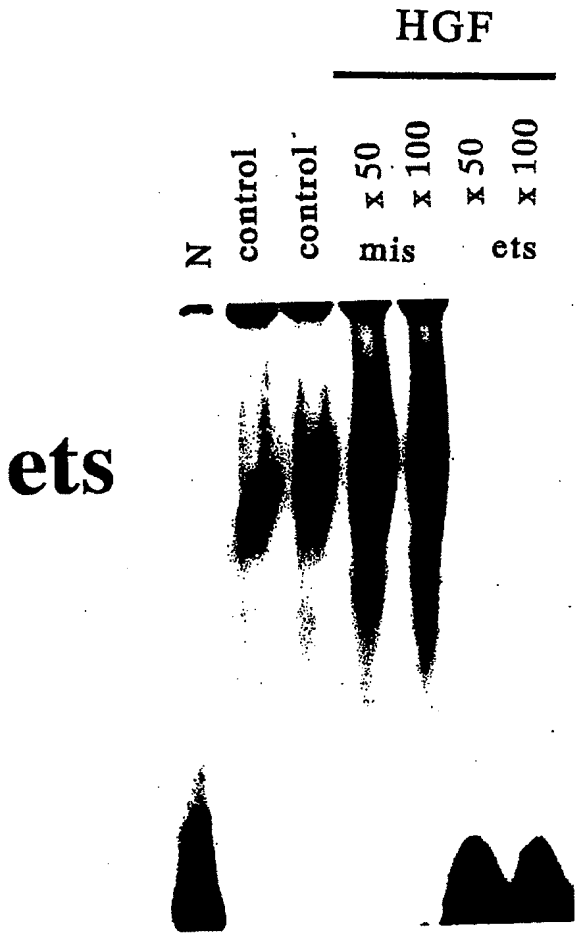
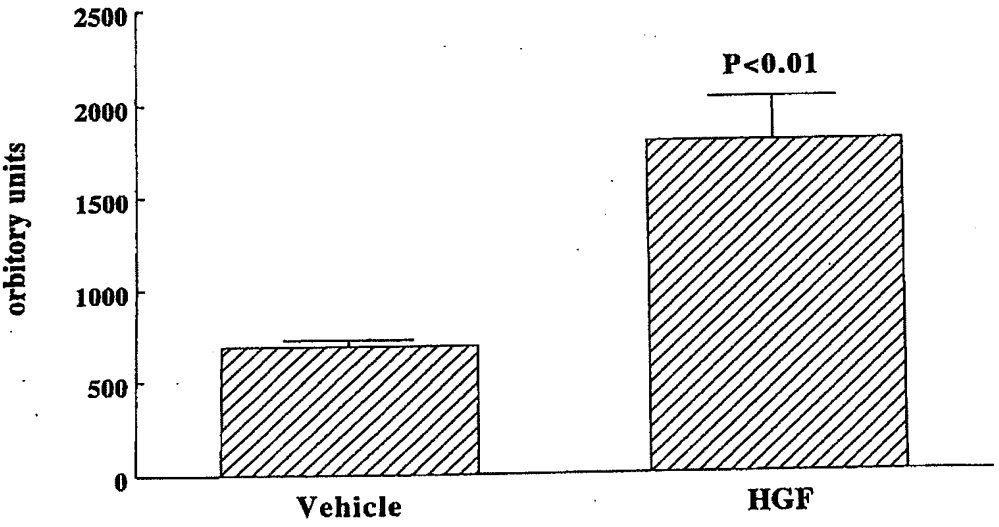
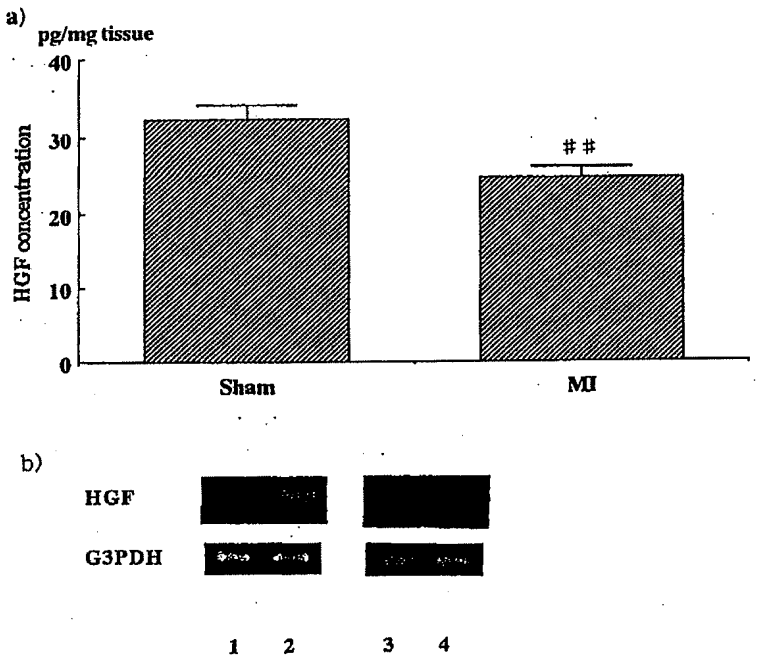
30
20
10
0
Numebr of vascula
>10
<10
total
**
**
**
Control
HGF

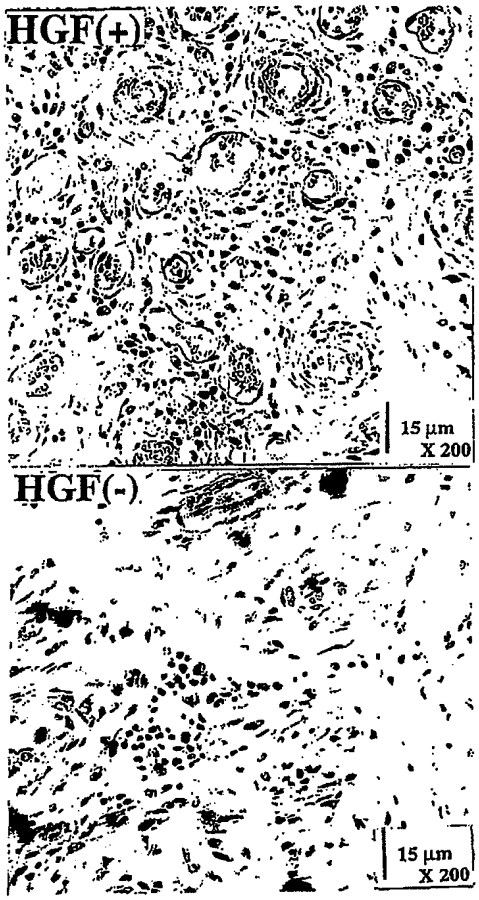

HGF
N
control
control
x 50
x 100
x 50
x 100
mis
ets
ets

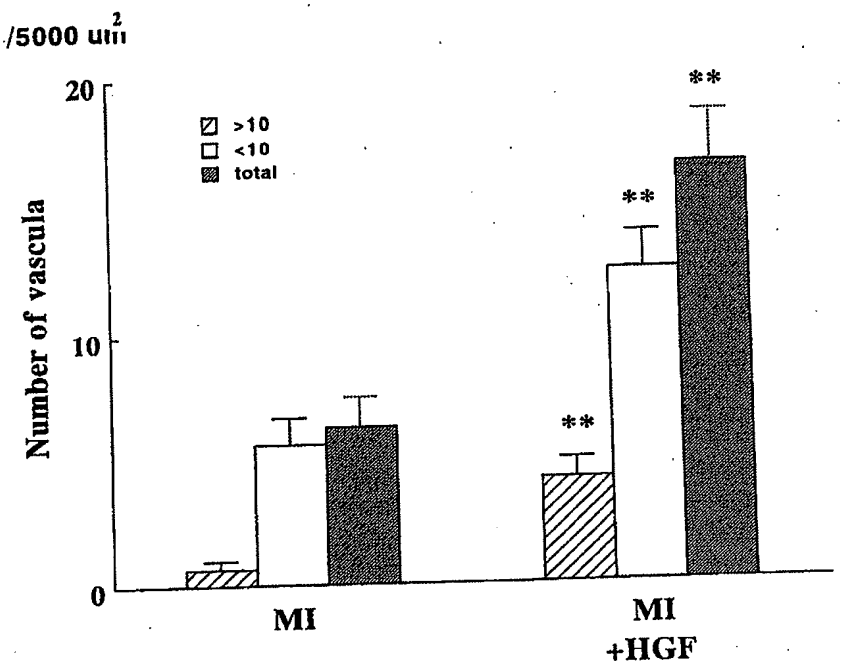
b)

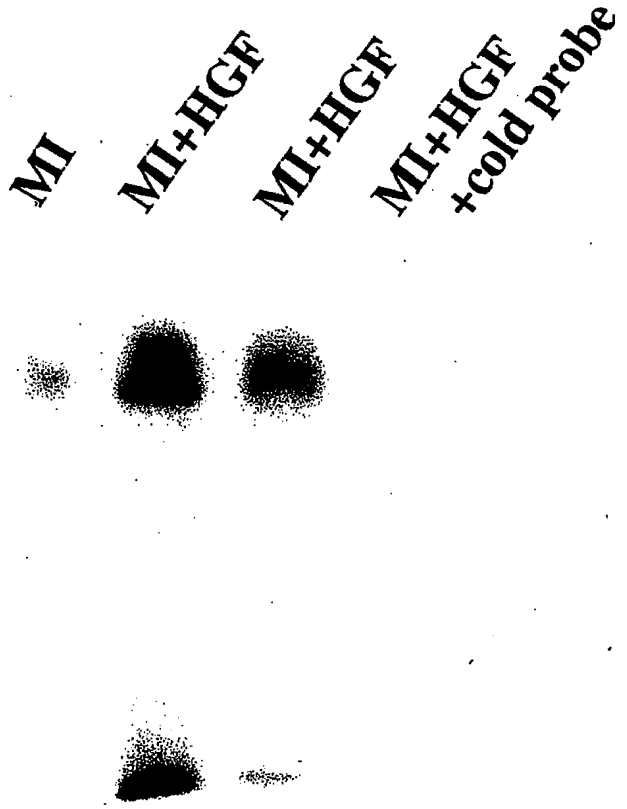


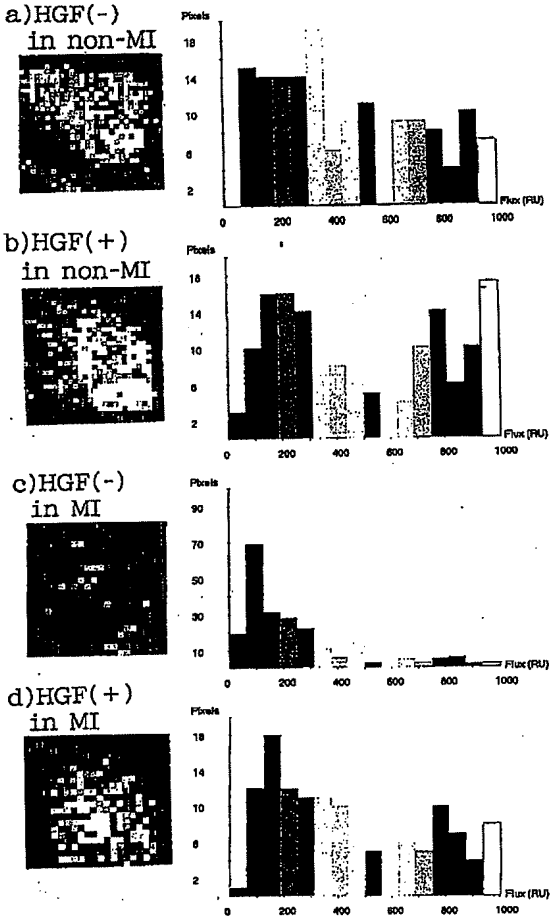

HGF
Control

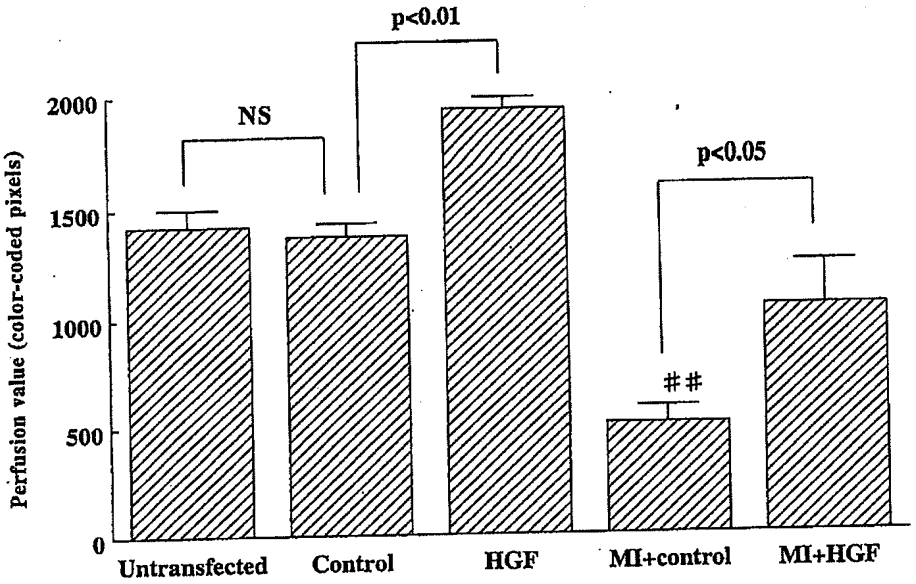

a)
pg/mg tissue
40
30
20
10
0
HGF concentration
# #
Sham
MI
b)
HGF
G3PDH
1
2
3
4


HGF(+)
15 μm
X 200
HGF(-)
15 μm
X 200


/5000 um²
20
10
0
Number of vascula
>10
<10
total
**
**
**
MI
MI
+HGF


MI
MI+HGF
MI+HGF
MI+HGF
+cold probe


a)HGF(-)
in non-MI
b)HGF(+)
in non-MI
c)HGF(-)
in MI
d)HGF(+)
in MI
Pixels
Flux (RU)
0
200
400
600
800
1000
2
6
10
14
18
30
50
70
90


p<0.01
NS
p<0.05
##
2000
1500
1000
500
0
Perfusion value (color-coded pixels)
Untransfected
Control
HGF
MI+control
MI+HGF